Functional dissection of ParB homologue and global regulatory protein KorB of RK2

by

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ABSTRACT

RK2 is a low copy number plasmid responsible for spread and maintenance of important properties (including antibiotic resistance and degradation functions) among bacteria. Gene expression in RK2 is controlled by cooperativity among four repressors (i.e. KorA, KorB, KorC and TrbA) to tightly regulate replication, stable inheritance and conjugative transfer functions. KorB (358 aa) has dual roles as a global regulator and as an active partitioning protein. This study focuses on its role as a global regulatory protein and its interaction with DNA, RNAP and other repressor proteins (e.g KorA and TrbA) of RK2. It is shown for the first time that DNA binding by negatively charged protein KorB (-21) is modulated via a balance of charge in the internal region from aa 235 to 245. KorB binds O_B and silences the genes around, showing that KorB can spread. TrbA and KorA bound to DNA adjacent to KorB do not block gene silencing by KorB and indeed potentiate its repression, suggesting that KorB can spread past DNA binding proteins and thus that they do not act as road blocks. The fact that KorB E237A, which is defective in silencing, cannot repress at a distance when alone but can do so in presence of TrbA, provides strong evidence of looping. The fact that KorA and TrbA do not potentiate gene silencing by E237A, but do potentiate its repression, indicates strongly that gene silencing is because of spreading instead of looping. Full length KorB is required for distal repression. However, only the region 225-255 aa is critical for proximal repression by KorB. The results suggest a model in which KorB organises DNA loosely over a long region through a wrapping in a way that can accommodate other regulatory proteins. This nucleoprotein complex may also be critical for plasmid partitioning.

CONTENTS

CHAPTER 1
Introduction

CHAPTER 2
Materials and Methods

CHAPTER 3
Role of the N-terminal domain of KorB in DNA binding

CHAPTER 4
KorB domains required for cooperativity with KorA and TrbA to regulate gene expression in RK2

CHAPTER 5
KorB amino acids critical for interaction with DNA and other repressor proteins in RK2

CHAPTER 6
KorA interaction with KorB in vitro

TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

ABBREVIATIONS

aa	Amino acid
A, C, G, T	Nucleotides: adenosine, cytosine, guanine, thymine
ADP	Adenosine diphosphate
APS	Ammonium persulphate
asRNA	Antisense RNA
ATP	Adenosine triphosphate
bhr	Broad-host-range
BSA	Bovine serum albumin
ccr	Central control region (operon)
CTD	C-terminal domain
D-plasmid	Degradation plasmid
DNA	Deoxyribonucleic acid
DNA Pol	DNA Polymerase
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamino-tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
H-NS	Heat-stable nucleoid-structuring protein
HTH	Helix-turn-helix motif
IHF	Integration host factor
Inc	Incompatibility (group)
IPTG	Isopropyl-β-thiogalactopyranoside
IS	Insertion sequence
LB	Luria-Bertani medium
NEB	New England Biolabs
NTP	Nucleotide triphosphate
O_A	RK2 (IncP-1) KorA protein binding site
O_B	RK2 (IncP-1) KorB protein binding site
OD_x	Optical density at x nm wavelength

o/n	Overnight
orf	Open reading frame
oriV	Origin of vegetative replication
par	Partitioning
PNK	Polynucleotide kinase
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
tacp	*tac* promoter
rbs	Ribosome binding site
rep	Replication
Ri	Repression index
RNA	Ribonucleic acid
RNAP	RNA Polymerase
SDS	Sodium dodecyl-sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDW	Sterile distilled water
Ta	Annealing temperature
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TEMED	N,N,N',N'-tetramethylene diamine
Tn	Transposon
Tris	Tris (hydroxymethyl) amino methane
tsp	Transcription start point
TTP	Thymidine triphosphate
X-gal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside
xylE	Catechol 2,3-oxygenase gene
WT	Wild type

Units

bp	Base pair
Ci	Curie

g	Gravity force
g	Gram
Da	Dalton
l	Litre
M	Molar concentration
rpm	Revolutions per minute
v/v	Volume of a substance per final volume
U	Unit of enzyme activity
w/v	Weight of a substance per final volume
^{o}C	Degrees Celsius
%	Percentage, grams per 100 ml final volume

Unit prefixes

k	Kilo, 10^{3}
m	Milli, 10^{-3}
μ	Micro, 10^{-6}
n	Nano, 10^{-9}
p	Pico, 10^{-12}
f	Femto, 10^{-15}

Chapter 1: Introduction

1.1 Plasmids

1.1.1 Plasmids

Plasmids are extrachromosomal DNA molecules. They can be circular or linear. They are capable of replicating autonomously as they have their own replication origin. They are non-essential for normal cell growth. Plasmids can confer many functions on their host cells, for example resistance to antibiotics, metals, toxic ions etc. They can also encode enzymes that are capable of metabolism. They are used as a major tool in gene cloning and gene manipulation.

Plasmids can be divided into two groups on the basis of their conjugation function:

• **Conjugative plasmids:** This is a type of plasmid that encodes *tra* genes that can initiate conjugation and the transfer of plasmids to bacteria.

• **Non-conjugative plasmids**: This is a type of plasmids that are incapable of initiating conjugation but may get transferred along with conjugative plasmids.

Plasmids can also be divided on the basis of their function:

• **Fertility (F) plasmids:** This is a type of plasmid that can promote transfer of chromosomal DNA.

• **Resistance (R) plasmids:** This is a type of plasmid that encodes antibiotic resistance.

• **Col plasmids:** This is a type of plasmid that produces a bacteriocin which kills *Escherichia coli*

• **Degradative plasmids:** This is a type of plasmid that is involved in the degradation of toluene and benzoic acid, e.g. Tol plasmids.

• **Virulence plasmids:** This is a type of plasmid that is responsible for conferring the ability to cause disease in its host, e.g. tumor initiation in plants by Ti plasmids.

1.1.2 Plasmid Maintenance

Stable maintenance of plasmids depends on a number of functions that prevent the irreversible loss of the plasmid during cell growth and division. Basically there are four types of such mechanisms: copy number control to ensure a sufficient number of segregating units; multimer resolution system (mrs); post-segregational killing of plasmid-less cells (PSK); and active partitioning systems (Par) (Thomas, 2000).

Plasmids can form dimers and multimers as a result of an odd number of recombinations during or after replication and thus reduce the number of independently segregating units (i.e. segregational instability). Because dimers and higher multimers have more than one replication origin, they will be chosen more frequently and when chosen will give a larger increase in copy number per replication cycle. This will result in the appearance of dimers only cells, a phenomenon known as “Dimer Catastrophe” (Summers et al., 1993). To counteract this problem dimers/ multimers are converted into monomers by a chromosomal- or plasmid-encoded recombinase.

1.1.3 Plasmid multimer resolution system and postsegregational killing

Multimer resolution systems have been described for F plasmid (the *rsf* and the product of *D* gene) (Lane et al., 1986), the P1 plasmid prophage (the *loxp* site and Cre recombinase) (Austin et al., 1981), and ColE1 plasmid (the *cer* site at which the host-encoded Xer-recombinase acts) (Summers and Sherratt, 1984; Stirling et al., 1988a). The multimer

resolution system of RP4, which is indistinguishable from RK2 (Burkardt et al., 1979), is encoded in the *par* region that consists of five genes (i.e. *parA, parB, parC, parD, parE*) organised into two divergently transcribed operons (Eberl et al., 1994). The first operon contains *parC*, *parB* and *parA*, and the second operon consists of *parD* and *parE* (Roberts et al., 1994). Both operons are negatively autoregulated. Two promoters are arranged back to back within an intergenic region between *parC* and *parD*. The first operon, *parCBA* is involved in multimer resolution whereas the second operon, *parDE* promotes plasmid maintenance through postsegregational killing when the plasmid is lost (Robert et al., 1994). The ParE target is DNA gyrase, a key enzyme in DNA replication and maintenance of normal supercoiling density. The ParE protein is responsible for growth inhibition, while ParD neutralises its toxic activity (Roberts et al., 1994, Oberer et al., 1999) **(Figure 1.1)**. ParE (the product of *parE* gene) is a stable toxin and ParD (the product of *parD* gene) is an antidote (inhibitor) of ParE.This type of postsegregational killing system is called "proteic" as both components toxin and antitoxin are proteins.

1.1.4 Plasmid Partitioning

Plasmids can have high or low copy number. High copy number plasmids can control their copy number and stably maintain themselves in daughter cells by random partitioning. But low copy number plasmids need an active partitioning mechanism before cell division to ensure at least one plasmid per daughter cells.

Active partitioning systems normally require three components for their partitioning mechanism. They include the two proteins ParA and ParB, and a *cis* acting centromere-like site (whose function resembles the centromere during the eukaryotic mitosis) on which the partitioning complex is formed. ParB is a partitioning protein that recognises and binds to the

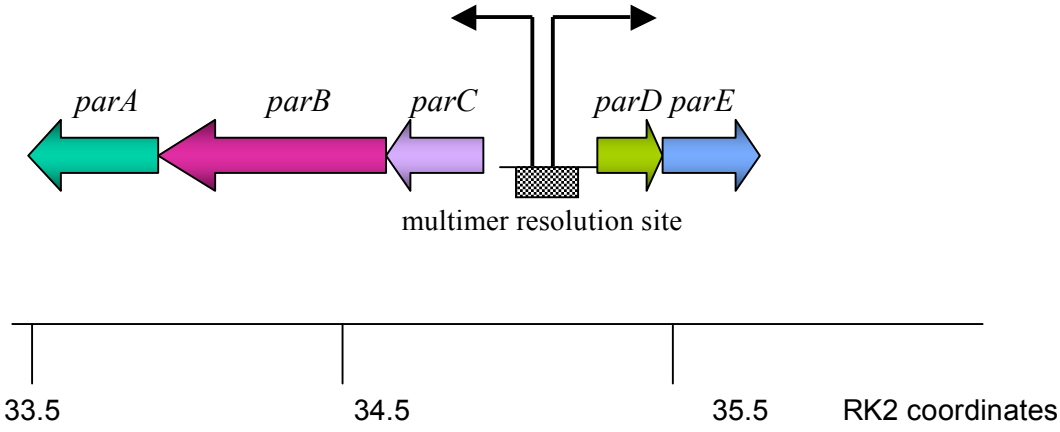


Figure 1.1: Structural organisation of the RK2 mrs/par region that encodes a multimer resolution system and a toxin-antitoxin system.

specific sequence along the DNA that forms the partition or centromeric site. ParB binding sites can be grouped at one locus (Hayakawa et al., 1985; Helsberg and Eichenlaub, 1986; Lane et al., 1987) or they can be scattered at different loci, as for example in N15 prophage (Ravin et al., 1999; Grigoriev and Lobocka, 2001) and in IncP-1 plasmids (Williams et al., 1993). The *parA* and p*arB* components of the active partitioning systems are normally encoded in one operon and are usually transcribed from an autoregulated promoter (Mori et al., 1989). The virulence plasmids pSLT (Cerin et al., 1989; 1993) and QpHI (Lin and Mallavia, 1994) are the only exceptions whose *parA* and *parB* genes are autoregulated by separate promoters.

The *par* loci have been divided into two main groups (i.e. Group I and II) on the basis of comparison of the amino-acid sequence of ParA and ParB, and the location of centromere-like site (Gerdes et al., 2000; Motallebi-Veshareh et al., 1990). Group I contains *par* loci in which ParA contains typical ATPase motifs that resemble the Walker motifs for NTP binding. Group I has been further divided into two subgroups (i.e. Ia and Ib) on the basis of homologies of ParB as well as the location of centromere. Subgroup Ia contains larger ParA and ParB proteins as compared to subgroup Ib. The centromere like site is located downstream of *parB* in subgroup Ia in contrast to subgroup Ib where it is present within the upstream promoter region. Subgroup Ia examples include the P1 and F plasmids as well as all putative chromosomal partitioning protein, whereas Ib examples include pTAR of *Agrobacterium tumefaciens* (Kalnin et al., 2000) and pRA2 of *Pseudomonas alcaligenes* NCIB 9867 (Kwong et al., 2001). Group II consists of *par* loci that encode an actin/hsp70-like ATPase (Bork et al., 1992) that are mostly present in plasmids and phages of Gram-negative bacteria such as example ParM of R1 plasmid (Moller-Jensen et al., 2002).

Active partitioning systems can be determinants of plasmid incompatibility (i.e. instability of coexistence of two different plasmids of the same group in the same host cell in the absence of any selection pressure). This phenomenon occurs in plasmids that have identical partitioning sites so that the partitioning apparatus is unable to distinguish between the two plasmids and thus results in a bacterial population containing only one type of plasmid (Austin and Nordstrom, 1990).

Active partitioning systems were discovered for the first time on the P1 (ParABS) and F1 (SopABC) plasmids. Knowledge about these systems has contributed greatly to the understanding of the active partitioning mechanisms (Nordstrom and Austin, 1989; Hiraga, 1992; Radnedge et al., 1996; Niki and Hiraga, 1997). Par homologues involved in active partitioning have also been reported for bacterial chromosomes i.e. *Pseudomonas aeruginosa, Pseudomonas putida* (Glaser et al., 1997; Gordon et al., 1997; Lewis et al., 2002), *Bacillus subtilis* (Ireton et al., 1994), *Caulobacter crescentus* (Mohl et al., 1997), and in the linear chromosome of *Borrelia burgdorferi* (Fraser and Claire, 1997). However, the active partitioning process is still not completely understood.

The active partitioning system of RK2 is very important for its survival and stable inheritance in a broad range of hosts. ParA and ParB homologues in RK2 are IncC, a putative ATPase (Motallebi-Veshareh et al., 1990; Batt, PhD Thesis 2008), and KorB, a specific DNA binding protein (Balzer et al., 1992) respectively. KorB and IncC proteins are highly conserved between IncP-1α and IncP-1β subgroups. They are encoded in one operon and KorB negatively autoregulate this operon by interaction with IncC protein. KorB and IncC are examples of Ib subgroup of *par* locus. IncC contains two Walker motifs; the Walker A motif and the Walker B motif, which is always found adjacent to the former (Motallebi-Veshareh et

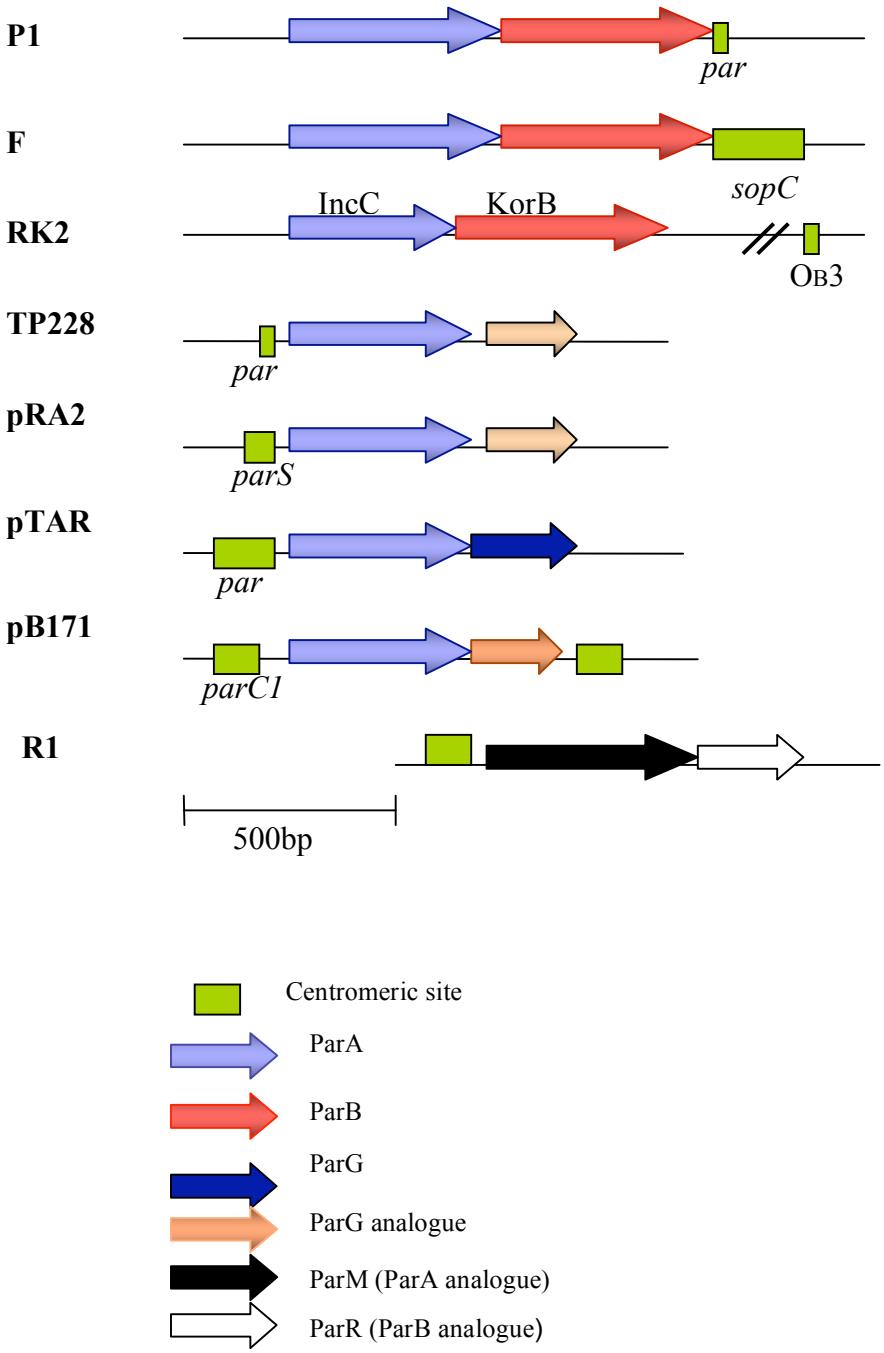


Figure 1.2: Genetic organization of *par* operons in different systems (Hayes and Barilla et al., 2006).

al., 1990; Koonin, 1993). The i*ncC* gene has two start codons and thus produces two types of IncC; IncC1 (364 amino acids) and IncC2 (259 amino acids). IncC1 potentiates the activity of global regulator KorB (Jagura Burdzy et al., 1999) whereas IncC2 is smaller than IncC1 and is involved in the active partitioning mechanism (Siddique and Figurski, 2002; Rosche 2000; Williams et al., 1993). IncC interacts with KorB (Rosche et al., 2000; Lukaszewicz et al., 2002). IncC1 resembles ParA homologues of plasmids, for example pM3 (IncP-9) of *Pseudomonas putida* and pFAJ2600 of *Rhodococcus erythropolis*, whereas IncC2 resembles ParA homologues of chromosomes in *Bacillus subtilis* and *Mycobacterium tuberculosis, Pseudomonas putida, Streptomyces coelicolor* (Hayes, 2000). IncC does not have a helix turn helix motif like other ParA homologues of plasmids (Bignell and Thomas, 2001; Adamczyk and Jagura-Burdzy, 2003), but recently it has been demonstrated that it does bind to DNA (Batt, PhD Thesis 2008). This indicates that IncC might have a different DNA binding motif.

IncC binds to 45 residues in the DNA binding region of KorB; from Ile174 to Thr218 (helices 3-6). This region is acidic in nature. IncC is highly basic (pIs › 10) and binds to the acidic region of KorB by Coulomb interactions (Khare et al., 2004). After binding to KorB, IncC is believed to enable the movement of plasmid/chromosome to the opposite poles (Rosche et al., 2000) due to conformational changes of IncC as it converts from the ATP-bound to ADP-bound form (Bignell and Thomas, 2001; Davey et al., 1997; Bouet et al., 1999; Quisel et al., 2000). It has been hypothesised that the ATP-bound form of IncC is responsible for the attachment of the partitioning components to the cell wall, whereas the ADP-bound form of IncC results in the dissociation of partitioning components from the cell wall (Adamczyk and Jagura-Burdzy, 2003).

1.1.4.1 ParA

ParA proteins are ATPases (Motallebi-Veshareh et al., 1990) that interact with ParB proteins and direct the partitioning complex to the proper position (Erdmann et al., 1999). They have the ability to hydrolyse ATP. They were discovered for the first time on P1 plasmid (Abeles et al., 1985). It has been suggested that the ParA-ATP complex might facilitate the attachment of the partitioning complex to the cell envelope and afterwards get converted into ParA-ADP complex and thus change the conformation (Bignel and Thomas, 2001). ParA removes ParB protein from the partitioning complex when present at low concentration (Bouet and Funnell, 1999) and results in an abnormal positioning of ParB loci as observed by immunofluorescence microscopy (Erdmann et al., 1999). However, the role of ParA protein is not fully understood yet.

1.1.4.2 ParB

ParB interacts with ParA and binds to the centromere-like sites along DNA to form a partitioning complex that interacts putatively with a specific cellular receptor that directs the partitioning complex towards the polar region of the host cell via an unknown mechanism. Purified ParB is able to stimulate ATPase activity, implying that ParA and ParB interact *in vivo*. Homologues of the plasmid partitioning protein ParB have been identified in a number of species.

ParB proteins have characteristics of dimerization, multimerization, DNA binding, transcriptional repression, spreading along DNA, gene silencing, interaction with ParA and other proteins (Bignell and Thomas, 2001). The C-terminal region contains the dimerization domain (Lobocka and Yarmolinsky, 1996) and deletion of 18 aa from C-terminal domain results in localization of Spo0J (ParB homologue) being lost. Thus the C-terminal domain is involved in the dimerization as well as localization of ParB protein in *Bacillus subtilis*.

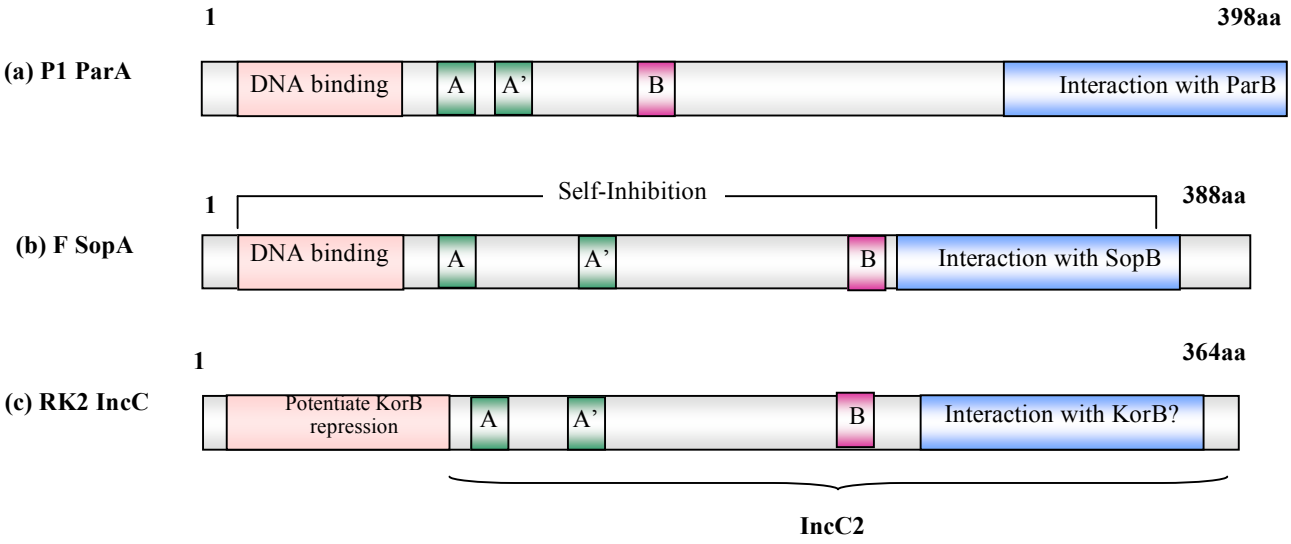


Figure 1.3: Comparison of ParA proteins (A,A` and B show ATPase motifs). (a) P1 (ParA), (b) F (SopA) –there are two regions responsible for interaction with SopB: 206-313 and 96-113 (Ravin *et al.*, 2003), (c) RK2 (IncC) –It has been proposed that the N-terminus of IncC is responsible for potentiation of KorB repression as this activity has only been observed for IncC1 (Kostelidou et al., 2000).

Figure 1.4: Sequence Alignment of Type I ATPases. Active sites are coloured: A motif (yellow), A' motif (orange) and B motif (pink) (Koonin, 1993).

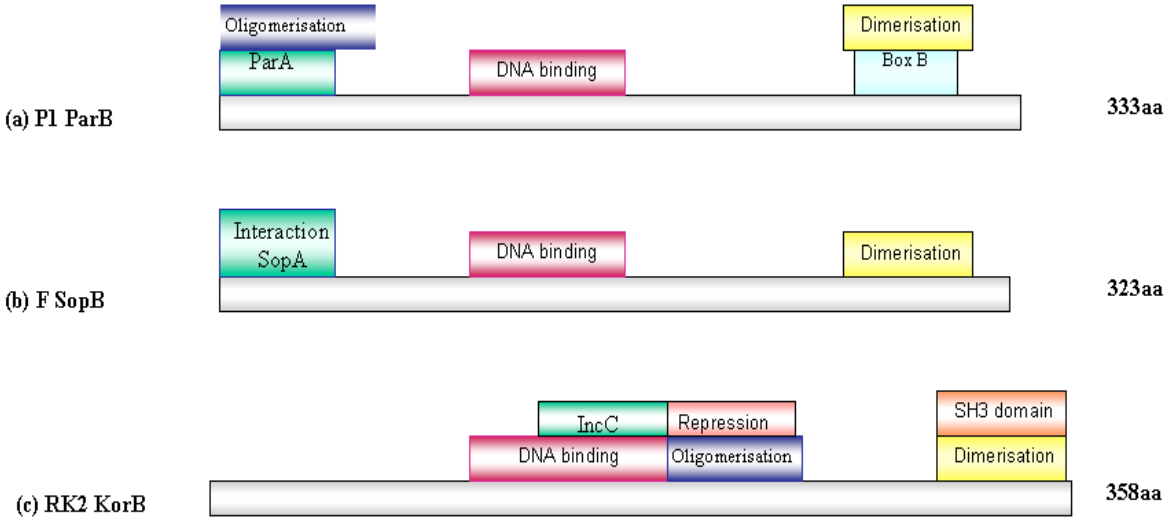


Figure 1.5: Plasmid ParB proteins**.**

(Pink shows SNA binding motifs for interaction with *parS* site, green shows region that interacts with cognate ParA and yellow shows dimerisation domain)

(a) ParB of P1, (b) SopB of F, (c) RK2 (ParB homologue KorB)-residues 151-218 are responsible for KorB's repression. Dimerisation mainly occurs at the C-terminus. The secondary oligomerisation domain at 174-218 is also required for interactions with IncC.

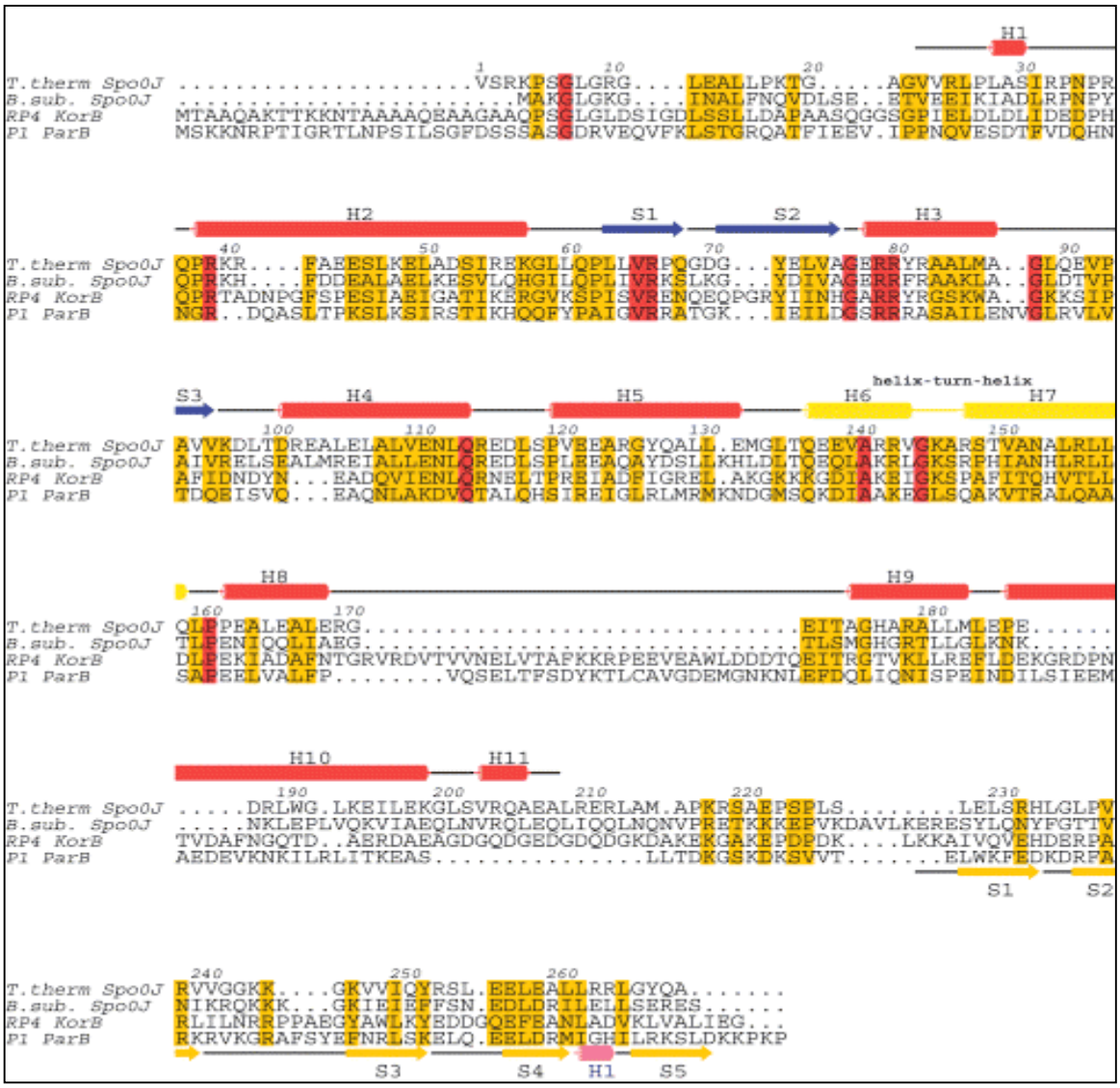


Figure 1.6: Sequence alignment of the ParB proteins. The secondary structure of KorB–C (PDB entry 1IGQ) is shown below the alignment. Identical residues are coloured red, and conserved residues are coloured orange (Leonard et al., 2005).

In the prophage of bacteriophage P1, the ParB protein binds to the repeated sequences *parS* (*cis* acting site) downstream of the *parB* gene sequence, and these sequences are known to exist downstream of the *parAB* operon in a variety of similar plasmids. Plasmids with mutations in the *cis*-acting coding region cannot be stably maintained (Bignell and Thomas, 2001).

In *Caulobacter crescentus*, ParB binds to the specific DNA sequences (*pars* sites) adjacent to the *oriC* (Mohl and Gober, 1997). This specificity of the binding of ParB is dependent on the dimerization domain in the C-terminal region as mutations in this region resulted in the loss of the ability of ParB to bind DNA specifically.

1.1.4.3 Centromeric sites

Centromeric sites consist of specific sequences to which ParB proteins bind to form a partitioning complex named as centrosome or segrosome. These sites are specific to each system in their sequence, number, length and position to ensure that only ParB from the cognate system would bind (Abeles et al., 1985; Hayes et al., 1994).

In eukaryotes, these sites act as attachment points for spindle microtubules to ensure accurate chromosome segregation during cell division. Plasmid centromere location is diverse. Most of the partitioning systems contain only one centromeric site located near the *par* operon (i.e. upstream or downstream) with the exception of RK2, containing 12 sites scattered over the whole plasmid. The centromeric site is present downstream of the operon with large Par proteins, and upstream of the operon with short ParA. The non-coding sequences upstream and downstream of the operon may also contribute to partitioning through the nucleoprotein filament of ParB with DNA but this phenomenon requires further investigation (Ebersbach and Gerdes, 2001; Yates et al., 1999). Plasmid centromeres can consist of direct repeats or

inverted repeats of DNA: P1 *parS* site consists of 80 bp (Bouet and Funnell, 1999), F plasmid *sopC* consists of 12 tandem repeats of 43 bp motif (Biek and Shi, 1994), pTAR *parS* consists of 13 heptameric repeats that are separated by integral helical turns (Kalnin et al., 2000), RK2 centromeric site consists of a 13 bp palindrome (Williams et al., 1998). ParB proteins bind to centromeric sites, wrap the DNA around themselves and result in topological changes in DNA which are crucial for its correct function (Hayes and Barilla, 2006). Centromeric sites have also been reported in many systems for their involvement in the autoregulation of the partitioning operon.

1.2 Partitioning Systems

Some of the partitioning systems are described in the following in order to understand more about ParB homologues and their role in active partitioning. This provides an important background to understand the nature of ParB homologue KorB in future experiments.

1.2.1 Bacteriophages P1 and P7

P1 and P7 are low copy number bacteriophages that are closely related and contain a large region of homology (Yun and Vapnek, 1977). Both replicate within *E. coli*. The partitioning system of these bacteriophages consists of ParA, ParB and a centromere-like site *parS* (Austin and Abeles, 1983; Gerdes and Molin, 1986; Ogura and Hiraga, 1983). This centromere like site is highly conserved between both plasmids (Radnedge et al., 1996) but species specificity is still present (Hayes and Austin, 1993).

The *parS* site (85 bp) consists of two types of repeats that are recognised by ParB: a heptamer, box A and a hexamer, box B **(Figure 1.7 and 1.8)**. Each ParB monomer recognises

and binds to both box A sequences (A2 and A3) by a central HTH region (present between 166 to 187 residues) , and a box B sequence by its C-terminus **(Figure 1.8)** (Surtees and Funnell, 2001).

The *parS* site also contains an integrative host factor (IHF) recognition sequence (Davis et al., 1990) that is thought to bind to this sequence and bend the DNA and results in increased ParB affinity for its binding sites. If IHF binding site within the P1 *parS* site is lost or damaged, the affinity of ParB for DNA is highly reduced. The spacing between the A and B boxes and IHF is very important for the partitioning function (Hayes et al., 1994). Consequently, the partition complex consists of *parS* wrapped around an IHF-ParB core (Funnell, 1991). The specificity between P1 and P7 is determined by two base differences of B boxes. The sequence of P1 specific B box is T**CG**CCA, whereas in P7 this sequence is T**TC**CCA.

ParB binds flexibly to a box sequence on a different DNA duplex as revealed from the structures of ParB bound to a short DNA oligonucleotide. This also explains how ParB can bind and form a bridge between the two arms of the *parS* site; it could also be a method for pairing and rotating adjacent plasmids and contacting them in a number of conformations prior to segregation. The C-terminal dimerisation domain has novel folds that lock together, forming an anti parallel β sheet and coiled coil structure. The C-terminus must dimerise in order to bind DNA (Schumacher and Funnell, 2005). ParB does not need all *parS* sites to bind DNA, but a motif on either side of the bend is required as demonstrated by deletion analysis of *parS.* ParB can bind DNA in different orientations by binding to various combinations of the box sequences. Consequently, the multiple box motifs in the *parS* site enable further binding of ParB dimers or pairing of adjacent plasmids (Vecchiarelli et al., 2007).

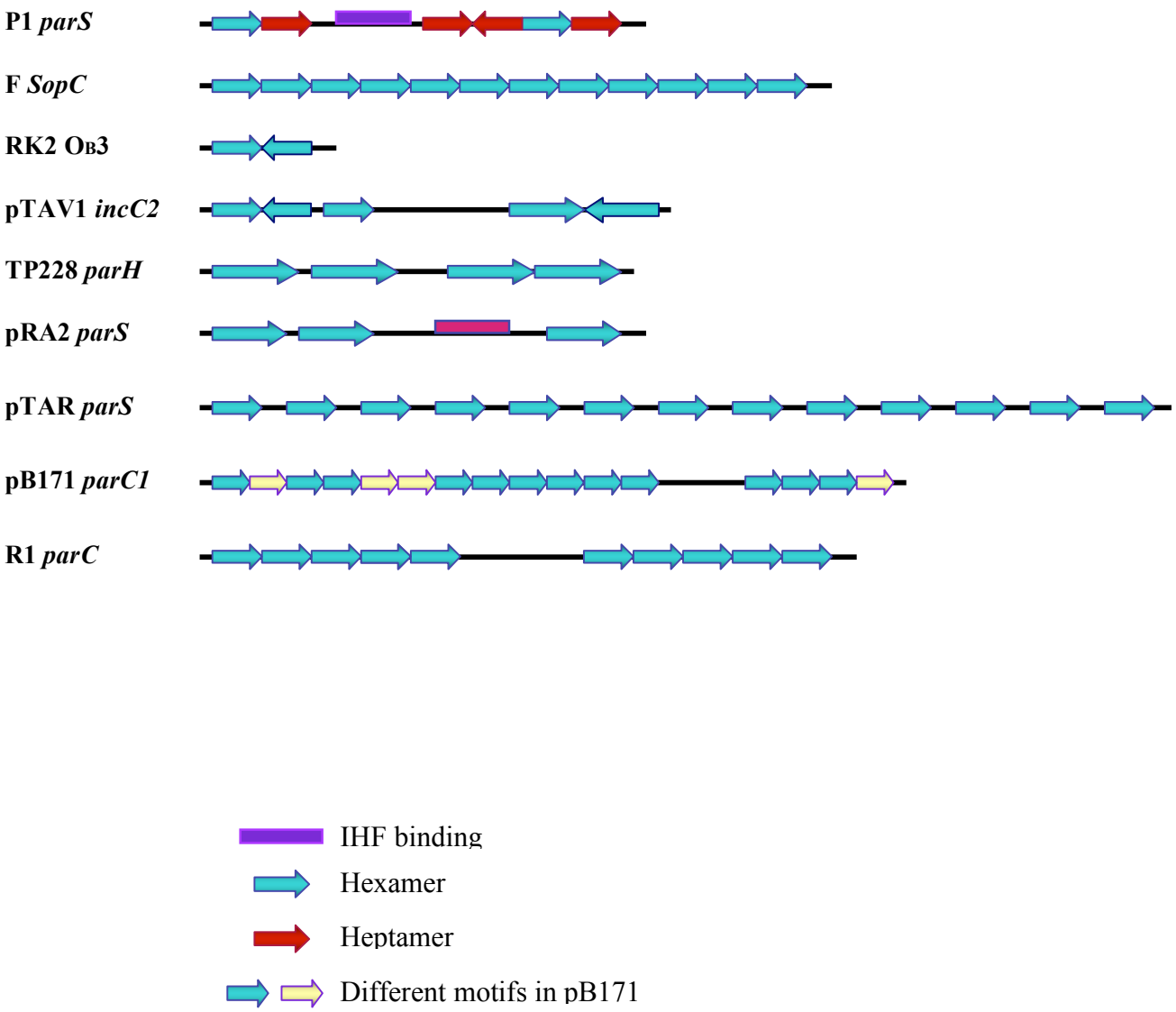


Figure 1.7: The diverse organisation of plasmid centromeres. Repeat sequences are shown by arrows (Hayes and Barilla, 2006).

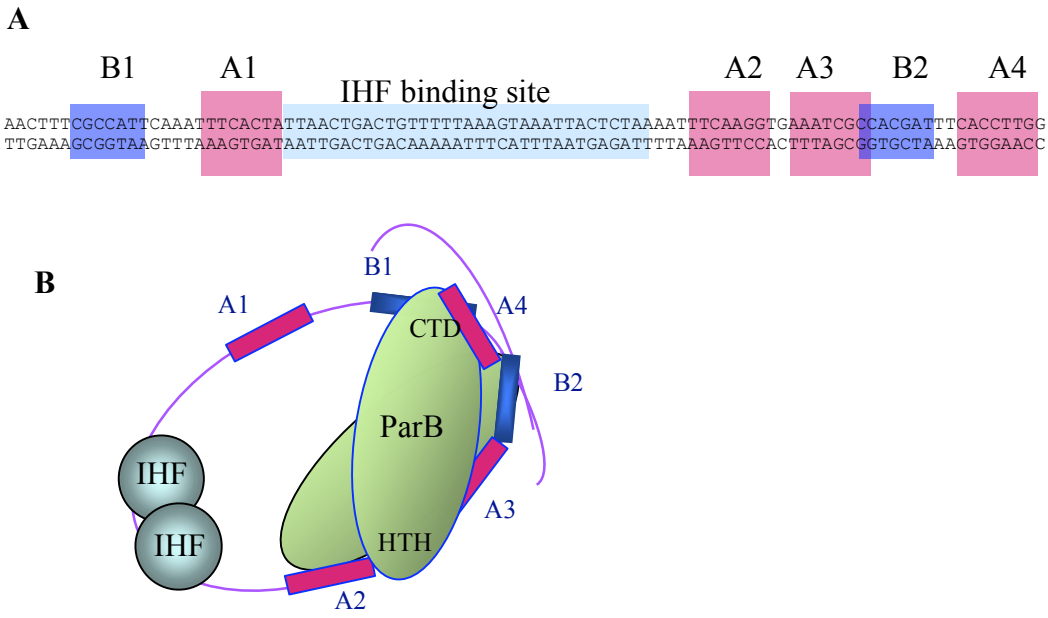


Figure 1.8: P1 partitioning complex. (a) Diagram of *parS*, showing ParB and IHF binding sites. (b) Model of the partition complex structure: the two HTH motifs bind A2-A3, while the dimerised C-termini hold the two box B sites together (Surtees and Funnell, 2001).

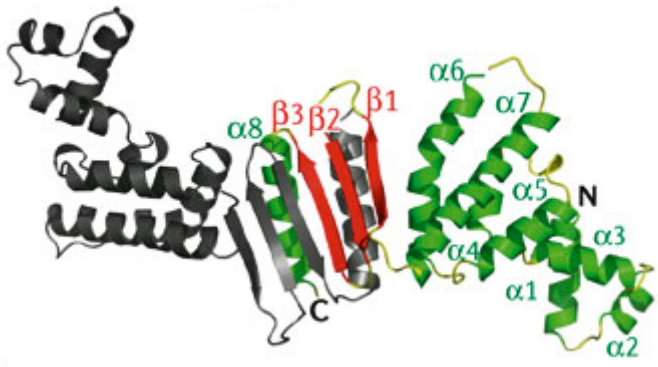


Figure 1.9: X-ray crystal structure of ParB (142-323 aa) bound to DNA (A3-B2 box). The HTH of each subunit binds to an A-box and each face of the dimerisation domain binds to a B-box. Monomers are shown in grey and colour. For the coloured monomer, β-strands and α-helices are red and green, respectively (Schumacher and Funnell, 2005; Hayes and Barilla, 2006).

The regulation of *par* operon is affected significantly when ParA and ParB are present in excess. The *parS* site also plays a role in the regulation of the *par* operon (Hao and Yarmolinsky, 2002). An excess of ParB does not interfere with the expression of ParA except when ParA is available in excess along with ParB. The specific ratio between ParA and ParB is very important: an imbalance may result in the destabilisation of the plasmid (Ables et al., 1985; Friedman and Austin, 1988; Funnell, 1988).

For the intracellular location of partitioning proteins and the partitioning complex, P1 plasmids were tagged with a *lac* operator where LacI-GFP repressor hybrid protein was provided in the cells. This is how copies of plasmids containing *lacO* cassettes were shown to be localised within the cell: in the new cell, plasmids were located at the midcell, whilst at cell division, they moved to the quarter cell positions (Gordon et al., 1997). A similar experiment with a ParB-GFP fusion showed localisation of ParB into discrete foci that corresponded with the position of the plasmid. Time-lapse microscopy has revealed that generally there is only one focus in the cell, early in the cell cycle. This single focus divides actively and migrates rapidly to the quarter-cell that marked the centre of the next cell generation. The speed of the P1 plasmid movement is approximately 50 times faster than the cell growth and five times faster than the *oriC* migration (Gordon et al., 2004). However, on replication, plasmid movement duplicates and travels to the quarter positions (Erdmann et al., 1999). The formation of ParB foci depends on the presence of *parS* only; however, ParA was required for the foci to segregate to the quarter positions, indicating that ParA is involved in the plasmid's movement. Time-lapse studies show that this ejection occurs immediately before segregation, implying that there could be coordination between partitioning and cell division. Li and Austin (2002), for example, found that partitioning defective ParB mutants remained at the midcell, produced aberrant cells. This suggests that P1 attachment to the midcell prevents cell division until the plasmid is segregated. ParA-GFP fusions, unlike ParB,

show more diffuse staining and did not produce the same bright foci, meaning that most of ParA is not bound at the partition site like ParB (Erdmann et al., 1999).

The ParA protein has weak ATPase activity, which is stimulated by ParB and non-specific DNA (Davis et al., 1992). P1 ParA belongs to the type I ATPase group; ATP binding domain is on the N-terminus of ParA (Gerdes et al., 2000). Functioning of ParA is dependent on its nucleotide binding motifs (Davis et al., 1996) and ATP hydrolysis is essential for partitioning (Fung et al., 2001). The form of NTP bound to ParA is also significant, affecting all aspects of its activities: repression (Davey and Funnell, 1994), conformation (Davey and Funnell, 1997), dimerisation (Davey and Funnell, 1994) and interaction with ParB (Bouet and Funnell, 1999).

ParA autoregulates the *par* operon by binding to its operator, *parOP*, which is present within the promoter region (Davis et al., 1992). ParA-ADP complex has higher affinity to bind DNA compared to ParA-ATP. It suggests that ATP hydrolysis prevents DNA-binding or triggers the release of ParA from the DNA (Davey and Funnell, 1994). The form of the nucleotide is believed to affect ParA by altering its conformation, in this case aiding DNA binding by promoting dimerisation (Davey and Funnell, 1994). Conversely, ParA's ability to interact with the partition complex is promoted by ATP. The non-hydrolysable ATPγS form also supports formation of the ParA-ParB-IHF complex, demonstrating that ATP hydrolysis is not required for this interaction (Bouet and Funnell, 1999).

Although ATP hydrolysis is believed to be the driving force for DNA movement, this data indicates that ATP hydrolysis plays an additional role: altering the function of ParA by controlling the ATP-ADP switch. Bouet and Funnell (1999) proposed a model to explain this (**Figure 1.10**). They suggest that cells in exponential phase have a higher concentration of ATP and most of the ParA binds nucleotides in this form. Consequently, ParA is recruited to

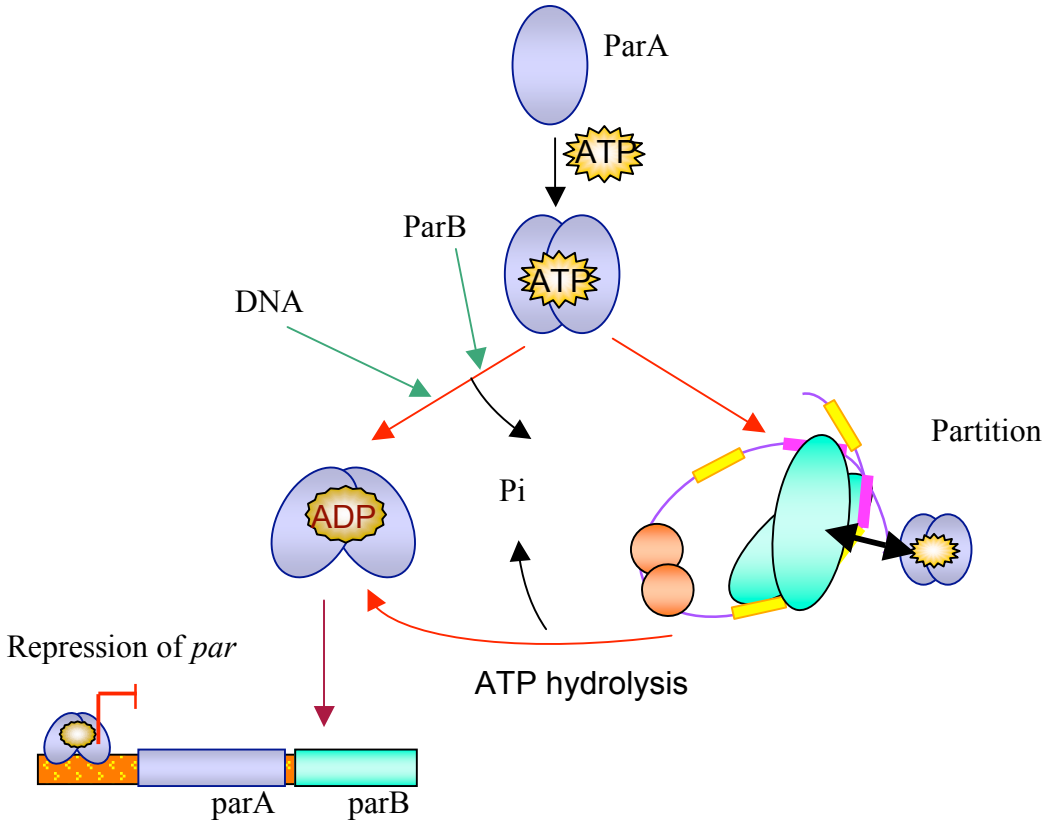


Figure 1.10: Model of ParA switch (Bouet and Funnell, 1999).

the partition complex, where ATP hydrolysis contributes to plasmid partitioning. This converts ATP to its ADP form, which promotes dimerisation of ParA, forming a repressor. ParA mutations can result in a significant decrease in the plasmid copy number because of disturbance in segregation of the plasmid. This results in a condition called a Par^{PD} phenotype. It could be that in such situations the mutant ParA is unable to separate aggregates of plasmids formed by ParB and thus all the plasmids go to one cell. This is an argument for plasmid pairing during segregation (Fung et al., 2001; Youngren and Austin, 1997).

1.2.2 F Plasmid

F plasmid is a low copy number plasmid (1-2). Its partitioning locus consists of *sopA* and *sopB* genes and the *cis*-action loci called *sopC* (Ogura and Hiraga, 1983).

sopC consists of 12 tandem imperfect repeats of 43 bp that are sufficient for the formation of partitioning complex (Biek and Shi, 1993; Lynch and Wang, 1994; Mori et al., 1986; Yates et al., 1999). The role of *sopC* in the enhancement of repression of the *sopA-sopB* operon is unclear. It was found to increase repression in the presence of SopA and SopB by 3.4 fold, but has no effect on expression levels in the absence of these proteins. *sopC* is thought to affect repression through binding to SopB, since *sopC* has no effect on repression when SopB is mutated (A183T); a substitution which prevents it from binding to *sopC*, but not to SopA. It is also shown that the interaction between the *sopC*-SopB complex and the SopA-operator complex might allow plasmid coupling **(Figure 1.11)** that would result in plasmid pairing; the first hypothesized step of partitioning (Yates et al., 1999).

SopB binds as a dimer to twelve tandem repeats of a 43 bp sequence within *sopC*, to form the partitioning complex. Once bound, SopB wraps around DNA in a right-handed coil, forming local, constrained, positive supercoils, which results in the reduction of overall negative

supercoils within mini-F plasmids. It is thought the *sopC* locus is wrapped around a protein core, consisting of several SopB proteins and also some host-encoded proteins. Although only one SopB binding site is required for SopB to initiate nucleation, it is believed that the presence of several SopB binding sites within the *sopC* locus enhances this process (Lynch et al., 1994). This finding is similar to the observations made in P1, where IHF and ParB bind to the DNA, bending and wrapping it around the protein core (Davis et al., 1990).

SopA does not only play a role in partitioning but also acts as an auto-repressor by binding to four repeats (5'-CTTTGC-3') within the *sopAB* promoter region (Mori et al., 1989). However, this repression is weak, but cooperative interaction with both SopB (Mori et al., 1989) and *sopC* (Yates et al., 1999) results in the full control. SopA is hypothesized to be regulated through a self-inhibition domain. Its N-terminus is thought to form a complex with the C-terminus, masking the operator-binding domain and preventing repression. SopB increases repression by competing with the C-terminus for its N-terminal interaction domain, and breaking open this inhibitory complex (Ravin et al., 2003). This has also been noted for P1's ParA protein (Radnedge et al 1996). SopA possesses type I ATPase activity (Gerdes et al., 2000). Mutation in the ATP binding site of SopA (replacement of lysine by glutamine at 120 residual position) results in the loss of partitioning function.This effect was even stronger when lysine was replaced by arginine. It shows that the integrity of the structure of the ATP binding domain of SopA is crucial for its proper functioning (partitioning) (Libante et al., 2001). Since addition of SopB did not increase the level any further, it is believed that SopB functions through this region of SopA. In the P1 system, it is supposed that ParB enhances repression by increasing ATP-hydrolysis (Davey and Funnell, 1997). However, SopA mutants (K120R and K120Q) were deficient in hydrolysis and it is believed that SopB must act by altering SopA's conformation; circular dichromism showed that the mutant proteins had an

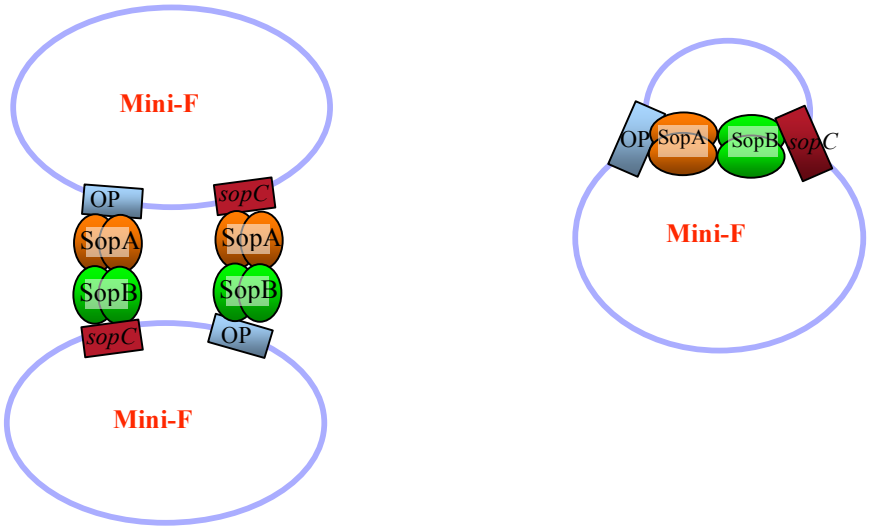


Figure 1.11: Theoretical interaction of the two complexes (*sopC*-SopB with SopA-operator), resulting in plasmid pairing in *trans* or intramolecular loops in *cis*. Adapted from Yates et al, 1999.

altered 3D structure in the presence of ATP/ADP. SopA has been shown to polymerise in the presence of ATP by forming long filaments, which extend at the same rate as plasmids are partitioned *in vivo.* By adding the *sopC/*SopB nucleoprotein complex, SopA radiates out from this complex, resembling the mitotic spindles of eukaryotes. This is theorized to be important in positioning the plasmid at the midcell prior to segregation (Lim et al., 2005). SopA polymerization is inhibited by the presence of DNA, which is relieved by DNA binding proteins including SopB. This is how improper polymerization of SopA is controlled (Bouet et al., 2007). Like other ParA proteins, SopA also oscillates from pole to pole by assembling near cell poles (at nucleoid tip) and dispersing and moving to the opposite pole, forming a low density filamentous structure in between (Hatano et al., 2007*)*. After an oscillation phase, the plasmid DNA splits into two foci at one of the cell poles and then one of these foci migrates to the opposite pole (Hatano et al., 2007).

Over-expression of Sop proteins results in the destabilization of the plasmid carrying the *sopC* locus (Mori et al., 1989; Ogura et al., 1990). Excess SopB can result in IncG incompatibility (Kusukawa et al*.,* 1987) due to gene silencing (Lynch and Wang, 1995) or multimerisation of the plasmid. This over-expression is counteracted by SopA, which could imply that SopA disrupts the extensive SopB-DNA complex, enabling proper segregation of the paired plasmids (Bouet et al., 2006). On the other hand excess SopA causes IncI incompatibility (Ogura et al., 1990) as it reduces the linking number of the plasmid that is opposite to what SopB does which was hypothesised to be ATP dependent. This means that SopA interferes with the stabilization of the partitioning complex either by binding to free SopB to prevent its binding to *sopC*, or directly interacting with SopB in the partitioning complex by hindering its DNA binding activity, removing it from *sopC*, or by stopping SopB from wrapping the DNA and forming a nucleoprotein complex. It has been hypothesized that partitioning is regulated by SopA's ATPase activity, which disrupts the interactions between adjacent SopB proteins

bound at *sopC* so that they interact with SopB on the opposite plasmid, and subsequent disruptions of these interactions result in segregation (Lemonnier et al., 2000).

1.2.3 R1 Plasmid

The partitioning system of R1 plasmid was investigated for the first time by the insertion of the expected *par* region. The partitioning system of plasmid R1 consists of two proteins: ParM (motor protein) and ParR (repressor), and a *cis* acting site *parC* that acts as a centromere. In contrast to the partitioning system of P1 and F plasmids, the centromere-like site in the R1 system is located upstream of the *parMR* region.

The *parC*, centromere-like site consists of about 160 bp located upstream of the *parMR* operon. It is composed of two sets of five 11 bp repeat boxes that flank the *parM* promoter. All of the ten iterons are required for ideal stabilisation of the plasmid (Dam and Gerdes, 1994; Jensen et al., 1994; Jensen and Gerdes, 1997; Breuner et al., 1996).

ParM (motor protein) belongs to a superfamily of ATPase that include actin and MreB (Bork et al., 1992). ParM is different from the Walker box family of ATPaes to which ParA homologues of other systems belong. According to electron microscopic studies ParM polymerises to form actin-like filaments that span throughout the cell. These filaments of helical appearance consist of several parallel protofilaments and their formation is dependent on ParR (ParB homologue) being bound at *parC*. These facts have lead scientists to propose that the partitioning complex stimulates ParM polymerisation by acting as a nucleating point (Moller-Jensen et al., 2002). The structural analysis of ParM has shown that it is very similar to F-actin (Van den Ent et al., 2002). ParM filaments are very dynamic as they are involved in both polymerisation (in the presence of ATPs and Mg^{+2}) and depolymerisation (in the

presence of hydrolysed ATP) that might result in an allosteric changes in ParM's structure (Moller-Jensen et al., 2002).

ParR (repressor protein) autoregulates *parMR* operon, which is how it distinguishes itself from ParB homologue of F and P1 plasmids (Jensen et al., 1994). Overexpression of ParR and/or ParM can lead to the destabilisation of the plasmid (Jensen et al., 1994). ParR binds to *parC* and forms a partitioning complex. Electron microscopy has shown that partitioning complexes are paired, possibly due to the ParR dimerisation/protein-protein interactions. Plasmid pairing always increases in the presence of ParM-ATP complex as well as in case of supercoiled DNA. This is why it has been proposed that plasmid pairing might be the first step in plasmid partitioning. However, significant evidence has only been found for R1 (Jensen et al., 1998). The electron microscopic studies of ParM protein have shown that it polymerises to form actin-like filaments that span the entire cell (Moller-Jensen et al., 2002), but unlike actin, the bidirectional polymerization of ParM is symmetrical (Garner et al., 2004). ParM filamentation was dependent on ParR being bound on *parC,* indicates that the partitioning complex stimulates it, possibly by acting as a nucleation point.

The R1 plasmid segregation model has been presented already. According to this model the plasmid replicates first at the midcell by the replication factory. Then plasmids are paired by dimerisation of ParR bound at *parS.* The paired partition complex is a nucleation point for the polymerisation of ParM into filaments. Further addition of the ParM-ATP to the poles drive plasmid segregation, and hydrolysis of ParM-ATP at midcell causes depolymerisation. After cell division, the cell cycle is repeated (Moller-Jensen et al., 2002).

1.2.4 TP228 plasmids and type Ib systems

TP228 is a multidrug resistant, low copy number plasmid found in *E. coli*. Its partitioning locus consists of two genes, *parF* and *parG* and the centromere-like sequence lies upstream of the *par* locus. These systems are type Ib; in some plasmids, including pTAR (Kalnin et al., 2000) and pRA2 (Kwong et al., 2001), the *cis*-site is present within the promoter region, and binding of the cognate ParB to this site autoregulates the operon.

ParF is more related to MinD subgroup than to the ParA proteins: only six of the fifteen conserved ParA amino acids are present in all members of the ParF group. These conserved residues are present in the ATP binding domain. ParF lacks the N-terminal helix turn helix domain, which is responsible for autoregulation in most of the ParA homologues. ParF homologues are present in VirC1 plasmids from *Agrobacterium*, pTAR from *A. tumifaciens*, pVS1 from *Pseudomonas aeruginosa,* and pB171 from *E. coli* (Hayes, 2000). ParF of TP288 is a multimeric protein whose self-association is affected by ATP (Barilla and Hayes, 2003), and it is speculated that ATP acts as a regulator of ParF polymerisation in a similar manner to MinD (Hu et al., 2002).

ParG is not a ParB homologue but it is essential for partitioning. Its homologues are found downstream of *parF* genes on other plasmids i.e pVS1 and pRA2 from Pseudomonas (Hays, 2000), pTAR from *A.tumifaciens* (Kalnin et al., 2000). ParG is a dimer in solution (Barilla and Hayes, 2003) and binds to the sequences upstream of the partitioning cassette, forming higher order oligomers, indicating spreading along the DNA similarly to the other plasmid systems (Rodionov et al., 1999). The protein has two regions: an N-terminal flexible region and a C-terminal ribbon-helix-helix (RHH). The RHH domain is required for DNA binding and is common amongst the Arc/MetJ family of transcriptional repressors, implying that ParG might have been a repressor, which has evolved to take part in plasmid partitioning

(Golovano et al., 2003). The flexible N-terminal region has two independent roles; similarly to other ParB proteins, it stimulates ATP hydrolysis by the cognate ParA protein (ParF) ; and it enhances ParF polymerisation (Barilla et al., 2007).

ParF interacts directly with ParG and potentiates its binding to DNA. ParF excess levels results in destabilisation of the partitioning complex (ParF-ParG-DNA). In contrast to the other ParF homologues, ParF interacts and participates in the partitioning complex formation in the absence of ATP, although ATP does enhance the process (Barilla and Hayes, 2003).

ParF has been shown to polymerise like other ParA proteins, a property that is thought to be involved in plasmid segregation. The purified ParF protein polymerises in the presence of ATP and ATPγS but not ADP, suggesting that ATP hydrolysis is not required. ParG also affects ParF polymerisation at a low ParG:ParF ratio. It has been speculated that ParG dimerises and interacts with ParF monomers in adjacent filaments, resulting in bundling. ParG was found to stimulate ATP hydrolysis, which is important in regulating ParF polymerisation *in vivo* and may explain why ParG destabilises polymers. But the way ParF polymerisation mediates partitioning is still unclear. It has been suggested that ParF might either pull or push the plasmid from the mid cell to the poles by anchoring to the host protein present at the cell pole. Alternatively, ParF filamentation could be coupled to polymerization of a cytoskeletal protein (Barilla et al., 2005).

1.2.5 *Bacillus subtilis* chromosomal partitioning system

The *Bacillus subtilis* chromosome partitioning system also contains ParA and ParB homologues, Soj and Spo0J respectively. Spo0J has a dual role, like KorB of RK2. It is involved in partitioning and in the regulation of sporulation. Spo0J is required for the initiation of sporulation which is the converse of the function of Soj (Ireton and Grossman,

1994). Soj, like other ParA proteins (Davis et al., 1992; Mori et al., 1989), also represses transcription but not of its own operon: it regulates expression of genes involved in sporulation, including *spoIIA, spoIIE, and spoIIG* (Quisel and Grossman, 1999). This is curious as Soj is a type Ib ATPase and does not have the N-terminal HTH motif of type Ia ATPases.

The chromosomal origins of *B. subtilis* and *E.coli* are in a defined position for most of the cell cycle in both species which is different to that of the plasmid studied. In new born cells, the origin is near to and oriented towards the cell pole; after replication, one copy of *oriC* resides at the cell pole, whilst the other moves to the midcell, which will become the new cell pole after cell division (Glaser et al., 1997; Gordon et al., 1997). However, insertion of Spo0J, Soj and *parS* (Spo0J binding site) into a plasmid, results in the movement of sister copies to the cell quarters in *E.coli,* which is more consistent with plasmid localisation patterns (Yamaichi and Niki, 2000).

Spo0J binds to a 16 bp sequence, *parS* (5′- TTGTTCCACGTGGAACAA-3′). There are ten *parS* sites located within 20° to either side of the origin of replication *oriC*, located at 0° on the 360° circular chromosome. These *parS* sites allow spreading of Spo0J over 20% (840 kb) of the chromosome and clustering around the origin region (Lin and Grossman, 1998), the compaction gained by extra *parS* sites may be less important in small plasmids.

The structure of Spo0J from *Thermus thermophilus* has been solved by X-ray crystallography **(Figure 1.12)** (Leonard et al., 2004). Each dimer has two HTH DNA binding domains, which bind to the major groove of DNA. Similarly to KorB from RK2, there are two dimerisation domains: a primary dimerisation domain at the C-terminus and second domain at the N-

terminus; this is hypothesized to mediate dimerisation during DNA binding, since in the absence of DNA, the N-terminal domain does not dimerise and has a slightly inhibitory effect on C-terminal dimerisation. The C-terminal is required for DNA binding along with N-terminal HTH, perhaps because C-terminal dimerisation is required to bring the DNA binding domain together into the correct orientation for binding (Leonard et al., 2005).

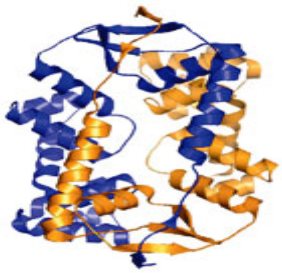


Figure 1.12: Crystal structure of Spo0J (1-222 aa). Monomers are shown in purple and gold. (Leonard et al., 2004; Leonard et al., 2005; Barilla and Hayes, 2006).

Spo0J and Soj molecular functions in partitioning are not well understood yet. Spo0J binds to *parS* sites and brings them together to form nucleoprotein complexes at each origin where Soj performs its ATPase function. It is not known how Soj and Spo0J contribute to the separation of sister origin regions, and it is possible that any of the effects of Spo0J spreading could contribute to this process (Breier and Grossman, 2007).

1.2.6 RK2 Plasmid

RK2 plasmid (60 kb) **(Figure 1.13)** is a relatively large, self-transmissible and relatively low copy number plasmid (i.e. 5-7 in *E. coli* per chromosome) (Figurski and Helinski, 1979), isolated in the Birmingham General Accident Hospital Burns Unit as an agent conferring

Carbenicillin resistance for *Pseudomonas sp* (Holloway and Richmond, 1973). It is the best studied example of an IncP α plasmid (Pansegrau et al., 1994; Thorsted et al., 1998). Most of the RK2 regions are highly conserved in R751, which is the representative of IncP β subgroup. RK2 has the ability to survive in various hosts' environments because of the autoregulation of the KorA and KorB regulons.

The RK2 active partitioning locus is located in the central control region, which was discovered first by Meyer and Hinds, (1982). The central control region of RK2 encodes KorA, KorB, IncC, KorF and KorG. KorB, a specific DNA-binding protein (Balzer et al., 1992), and IncC, a putative ATPase (Motallebi-Veshareh et al., 1990), are homologues of ParB and ParA partitioning proteins respectively **(Figure 1.14)**. Kor indicates the first identified function of these proteins, as products of **K**ill **O**ver**R**ide genes, suppressing the killing phenotypes (KilA and KilB) of *kilAB* genes. The ParA homologue called IncC was first identified as an incompatibility determinant that would cause displacement of another IncPα plasmid (Pansegrau et al., 1994). The *incC*, *korB* and *korA* are all expressed from the same promoter, which is autoregulated by two global repressors (i.e. KorA and KorB), which work together cooperatively to achieve better repression (Kostelidou et al., 1999).

The RK2 *cis* acting site has not been defined yet since there are 12 KorB operators binding sites named as $O_{B1\text{-}12}$ (consensus sequence: 5`TTTAGCGG/CGCTAAA3`) (William et al., 1993). It has been implied that KorB might act at more than one O_B site to pair and partition the plasmid, because the deletion of O_B3 made the plasmid unstable while detelion/mutation of O_B1 restored the instability (Williams et al 1998). It is unusual for plasmid ParB proteins to have so many binding sites but not for their chromosomal counterpart: *Bacillus subtilis* has 10 potential *parS* sites (Lin and Grossman, 1998) and *S. coelicolor* has 24 sites (Jakimowicz et al., 2002).

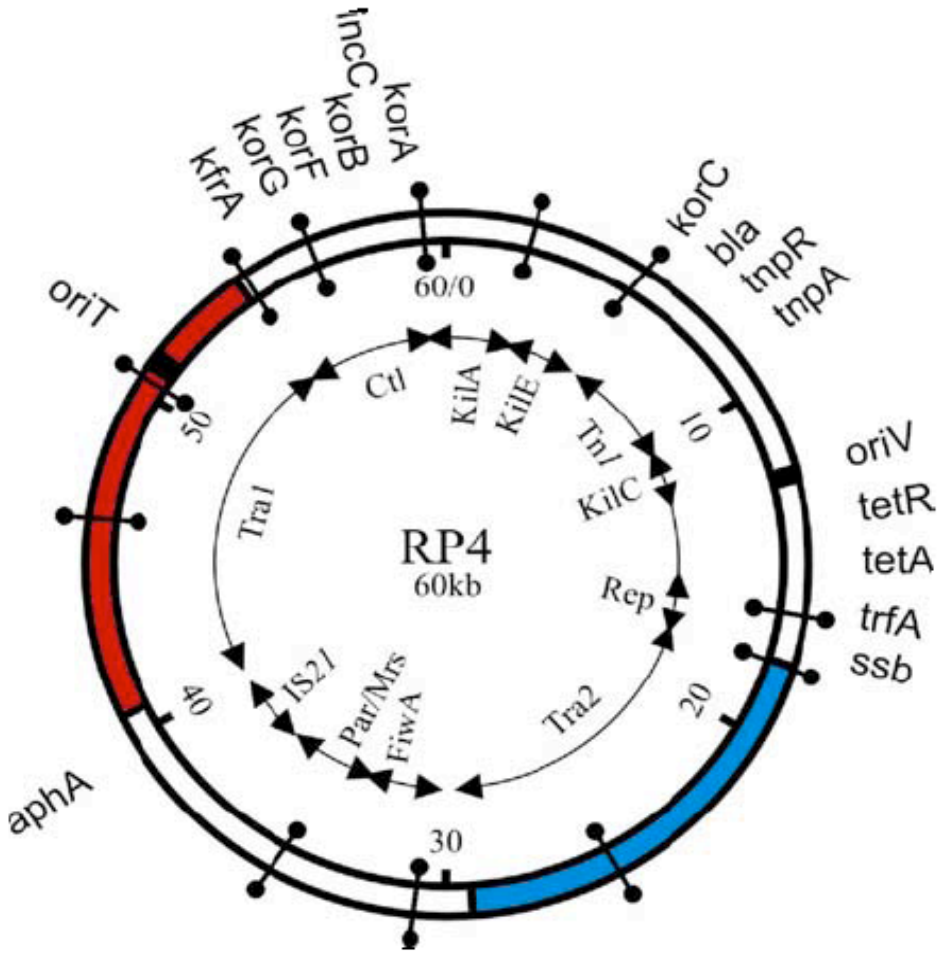


Figure 1.13: Physical and genetic map of plasmid RK2. The outer circle shows selected genetic loci and the inner circle shows blocks of related genes or distinct genetic elements like transposons and insertion sequences. The regulatory protein KorB binds to 12 operator sites (O_B1 to O_B12, consensus sequence 5′ TTTAGC$^{G}/_{C}$GCTAAA 3′) on the plasmid's genome (represented by dumbbells). (Adopted from Pansegrau et al., 1994; Balzer et al., 1992; Khare, 2004).

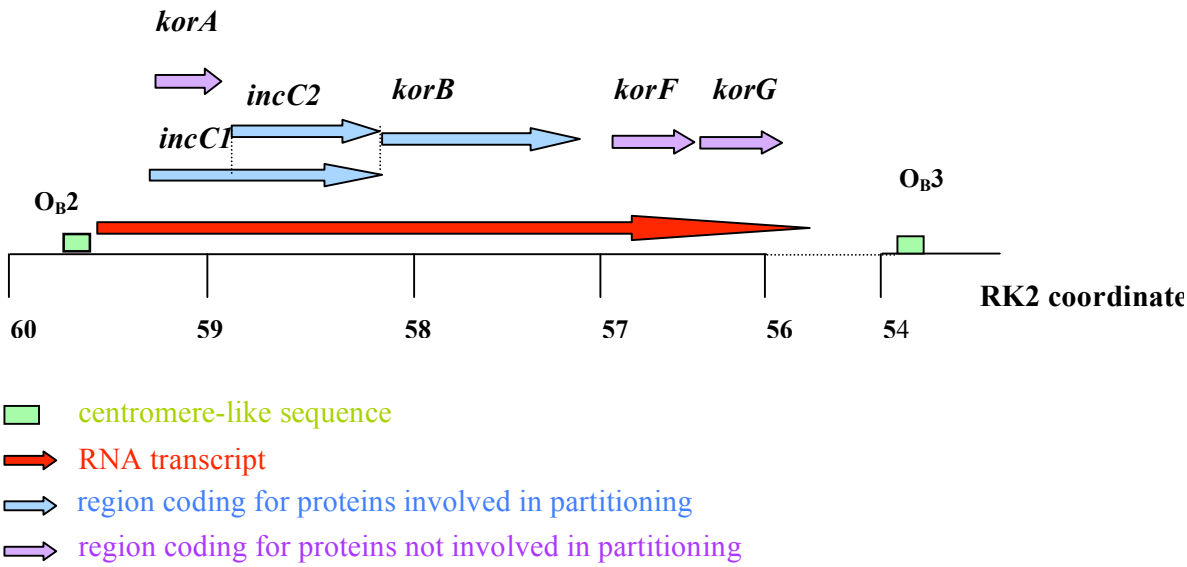


Figure 1.14: Central control region (*korAB* operon) of IncPα plasmids.

Arrows represents the genes. O_A and O_B are binding sites for KorA and KorB repressors. *IncC2* encodes for the small IncC2 polypeptide, which results from an internal translation start site in *incC*. *KorF* and *korG* encode for small basic proteins of unknown function. The *korA* gene is within the *incC* coding sequence but in a different reading frame; p, promoter (Adopted from Siddique and Figurski, 2002; Khare, 2004).

RK2 needs more than *inc*C, *korB* and *cis-acting* DNA sites to be fully stabilised. The additional genes include *korF, korG, kfrA*, *kla, kle, korC* and supplementary O_B include O_B1 and O_B2 (Rosche et al., 2000). Proteins encoded by the additional genes have been shown to be involved in the stabilization of paired partition complex (Rosche et al., 2000).

The *incC* gene is very interesting as it has two start codons which encode two IncC proteins: IncC1 (364 aa residues, *pI* 10.53 and a positive charge of + 11) and IncC2 (259 aa residues, *pI* 10.26 and a positive charge of + 7) (Pansegrau et al., 1994). IncC1 possesses a 100 aa N-terminal domain which is equivalent to that in other type Ia plasmid ParA proteins (i.e. P1 and F) to bind DNA (Friedman and Austin, 1988; Mori et al., 1989). IncC1 modulates KorB repressor activity *in vivo* and DNA binding *in vitro* (Jagura-Burdzy et al., 1999). It potentiates KorB repression at class I and II operators (Jagura-Burdzy et al., 1999) and stabilizes KorB – DNA complexes at all O_B sites except O_B3 (Jagura-Burdzy et al., 1999; Kostelidou and Thomas, 2000). It has been recently demonstrated that IncC possesses a DNA binding activity (Batt PhD thesis 2008). InC1 potentiates KorB binding to all O_Bs except O_B3 (Kostelidou and Thomas, 2000) and also plays a role in repression, whereas IncC2 is unable to do this and has been implied to be involved in partitioning (Jagura-Burdzy et al., 1999a). IncC1 has also been suggested to be host specific so it might be essential for partitioning in other bacterial hosts, similarly to KilE (Wilson et al., 1997). Both IncC proteins have an ATP binding motif but it has been shown that only IncC2 is sufficient for stability (William et al., 1998) in several gram negative bacteria (*E. coli, Pseudomonas aeruginosa, Pseudomonas putida, Agrobacterium tumefaciens and Acinetobacter calcoaceticus* (Siddique and Figurski, 2002).

1.3 ParB homologue KorB of RK2

1.3.1 Role of KorB

KorB is a DNA binding protein that belongs to ParB family. It ensures the segregational stability of low copy number plasmids, and its homologues are also involved in chromosomal segregation to various extents (Gerdes et al., 2000; Gordon et al., 2000; Jensen et al., 2000; Lewis et al., 2002). KorB is of particular interest because of its dual role in partitioning and global regulation.

1.3.2 KorB as a DNA binding Protein

KorB recognises and binds specifically to a 13 bp palindromic operator sequence (5`TTTAGCGG/CGCTAAA3`) present 12 times on the RK2 plasmid (i.e. O_B1-O_B12) (Balzer et al., 1994). Recently O_B sites have been discovered on the bacterial chromosomes of *Pseudomonas putida* and *Pseudomonas aeruginosa* (4 clustered O_B sites) that provide evidence of past integration of IncP 1 plasmids into bacterial chromosomes and also shows that IncP 1 plasmids are important for gene mobility (Chiu et al., 2004). The IncP β plasmids i.e. pB4, pADP-1 and pTSA (partially sequenced) contain 44 O_B sites that are identical to the consensus sequence. The twelve O_B sites (O_B1-O_B12) have been divided into three classes relative to their distance from the RK2 promoters. Class I operators (O_B1, O_B10, O_B12) lie immediately upstream (about 40 bp) of the -35 region of promoter (*korAp,trfAp, klaAp*), class II operators (O_B2, O_B9, O_B10, O_B11) lie 189 bp upstream or downstream of the transcription start point (*kfrAp*, *trbBp*, *trbAp*, *kleAp*). O_B10 is included in class I relative to *trfAp*, whereas it is in class II relative to *trbAp*. Class III operators (O_B3 to O_B8) lie more than 1 kb away from the nearest promoter **(Figure 1.15)**.

KorB binds DNA through a helix-turn-helix motif (HTH) (Theophilus and Thomas, 1987; Kornacki et al., 1987) like other ParB homologues (Gerdes et al., 2000; Bignel and Thomas, 2001; Khare et al., 2004). Helix turn helix is a well-characterised three-dimensional structure that allows many proteins to bind DNA. KorB binding sites (O_B) fall into three groups based on their binding affinity: Group A (highest affinity), Group B (medium affinity), Group C (lowest affinity) **(Table 1.1)**. Group A include O_B10, Group B include O_B3, O_B4, O_B5, O_B6, O_B7, O_B8, O_B9, and Group C include O_B2 and O_B6 (Kostelidou and Thomas, 2000). The highest affinity site O_B10 plays a crucial role in controlling *trfAp* (strongest promoter of *trb* operon) that transcribes genes for replication. This is how KorB plays a role in controlling the copy number of RK2 and the switch involved in the regulation of transferring genes through *trbAp* and *trbA* (Jagura-Burdzy and Thomas, 1997). The *trfA* promoter is the strongest promoter in the RK2 backbone and requires strict regulation by KorB bound at O_B10 (Kostelidou and Thomas, 2000; Thomas and Hussain, 1984). During plasmid establishment *trfAp* seems to be highly expressed and needs to be down regulated when the plasmid is established and repressor concentration rise. After plasmid establishment, transfer genes should be expressed at maximum and should be down regulated later. This is how orphan O_B sites may play a role in this down regulation. Therefore, two O_B sites (O_B4 and O_B7) belonging to class II O_B are at the lower end of the group B affinity (Kostelidou and Thomas, 2000). The sequences flanking consensus O_B sites also play an important role in determining the affinity of KorB binding. It shows that KorB also contacts the sequences flanking the O_B sites (Kostelidou and Thomas, 2000).

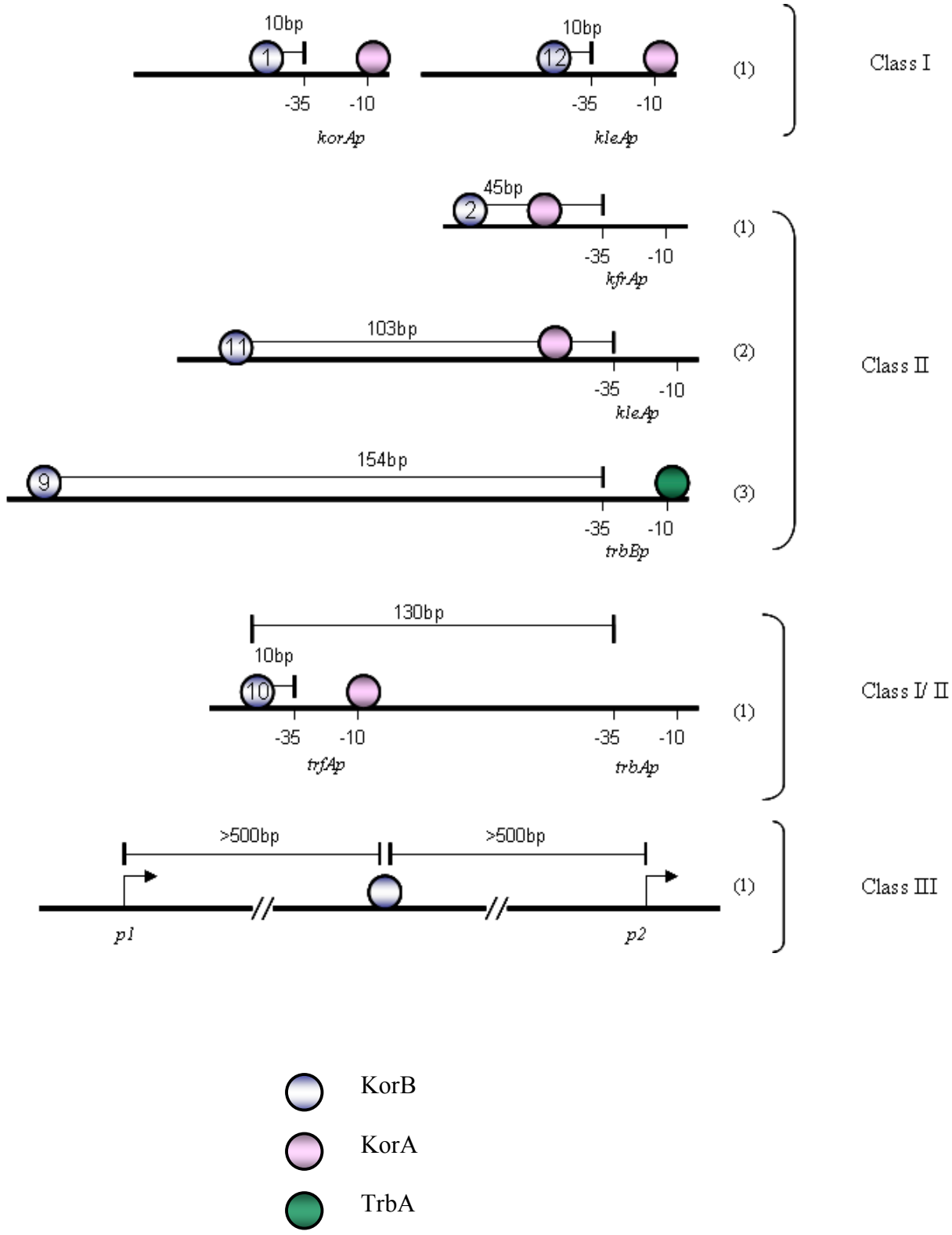


Figure 1.15: O_B classification based on its distance from the promoter (adapted from Bingle et al., 2005).

Table 1.1: The apparent affinities (K_{app}) of KorB for the 12 O_B sequences

Operator	Operator sequence (5′ to 3′ direction)	K_{app} (nM)	Group [a]	Class [b]
O_B1	ACACC TTTAGC^{C}/$_{G}$GCTAAA ACTCG	9.3 ± 0.6	B	I
O_B2	GGTTT TTTAGC^{G}/$_{C}$GCTGAA GGGCA	34.6 ± 1.9	C	II
O_B3	CCCTT TTTAGC^{C}/$_{G}$GCTAAA ACTCT	9.9 ± 0.9	B	III
O_B4	GCCGT TTTAGC^{G}/$_{C}$GCTAAA AAAGT	14.4 ± 1.1	B	III
O_B5	CGAGT TTTAGC^{C}/$_{G}$GCTAAA GGCGA	9.4 ± 0.9	B	III
O_B6	CGATT TTTAGC^{G}/$_{C}$GCTGAA ATCAG	32.4 ± 1.7	C	III
O_B7	TAGGC TTTAGC^{C}/$_{G}$GCTAAA CGGCC	13.8 ± 1.2	B	III
O_B8	GCTAC TTTAGC^{G}/$_{C}$GCTAAA ACATT	7.7 ± 0.9	B	III
O_B9	GCCGT TTTAGC^{G}/$_{C}$GCTAAA GAAGG	10.6 ± 0.9	B	II
O_B10	AGAAC TTTAGC^{G}/$_{C}$GCTAAA ATTTT	5.8 ± 0.4	A	I
O_B11	GCGGT TTTAGC^{C}/$_{G}$GCTAAA GTCCT	8.8 ± 0.6	B	II
O_B12	ACACC TTTAGC^{C}/$_{G}$GCTAAA ATTTG	8.0 ± 0.3	B	I

[a] Groups based on apparent affinity of KorB for each operator (Kostelidou and Thomas, 2000)

[b] Class based on relative location of the O_B sites with respect to promoters (Jagura-Burdzy *et al*., 1999b)

O_B carrying fragments were 300 base pair in length

The consensus sequence is given in blue and red underlined base is a mismatch. (Modified from Kostelidou and Thomas, 2000 and Khare, 2004).

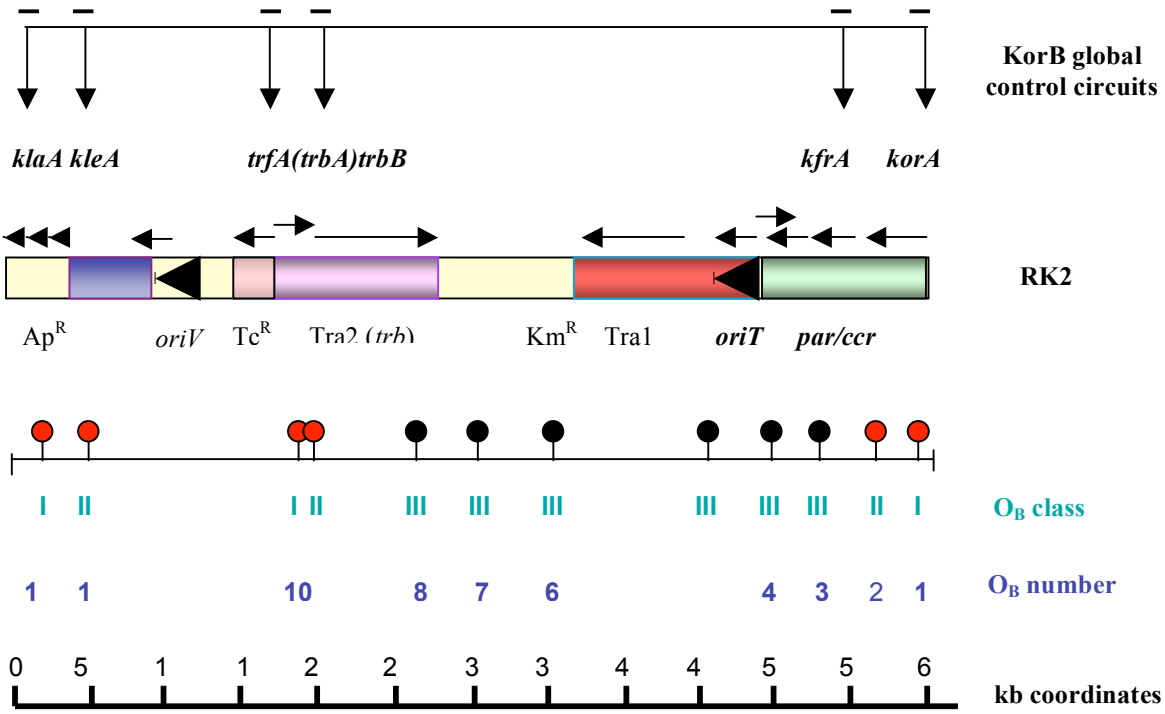


Figure 1.16: KorB global regulatory circuits. The effect of the protein on all promoters is negative (-21). Promoters and transcripts are indicated by arrows; the black triangles represent origin of replication (*oriV*) and conjugative transfer (*oriT*). Modified from Kostelidou and Thomas, 2000.

1.3.3 Classification of transcriptional repression by KorB

KorB exhibits three types of transcriptional repression: proximal repression, distal repression, and downstream repression. Proximal repression is when KorB is bound at class I operators, distal repression is when KorB is bound at class II operators, and downstream repression is when KorB is bound at class III operators.

KorB might use different mechanisms for proximal and distal repression. However, the mechanism for distal repression is not known yet. It has been shown that KorB interacts with RNAP differently at different promoters, e.g. by preventing binding of RNAP to the promoter sequences, or by inhibition of open complex formation (blocking of isomerisation from closed to open complexes), or by inhibition of promoter clearance – stopping RNAP from escaping from the promoter. At *korAp* KorB does not prevent binding of RNAP to the –10 and –35, but blocks transition from closed to open complexes **(Figure 1.17)**. At *trfAp* KorB acts by preventing RNAP to bind to DNA, at *trbAp* it may repress through locking RNAP molecules at *trfAp* ("road-block" mechanism) or by occlusion. *In vitro* DNase I footprinting experiments on *korAp* and *trbBp* suggested that KorB does not prevent RNAP simultaneous access to the promoter where it is bound. KorB can block transcription by preventing isomerization of the RNAP-DNA complexes from close to open (Williams et al., 1993).

It has been proposed in the past that KorB might bind to more than one binding site simultaneously in order to come in contact and thus interact with sequences flanking the operator and RNAP to form a multiprotein-DNA complex and prevent normal RNAP functioning (Jagura-Burdzy et al., 1999; Adhya et al., 1998). Homologues of KorB have been shown to repress by gene silencing mechanism in which a DNA binding protein binds and

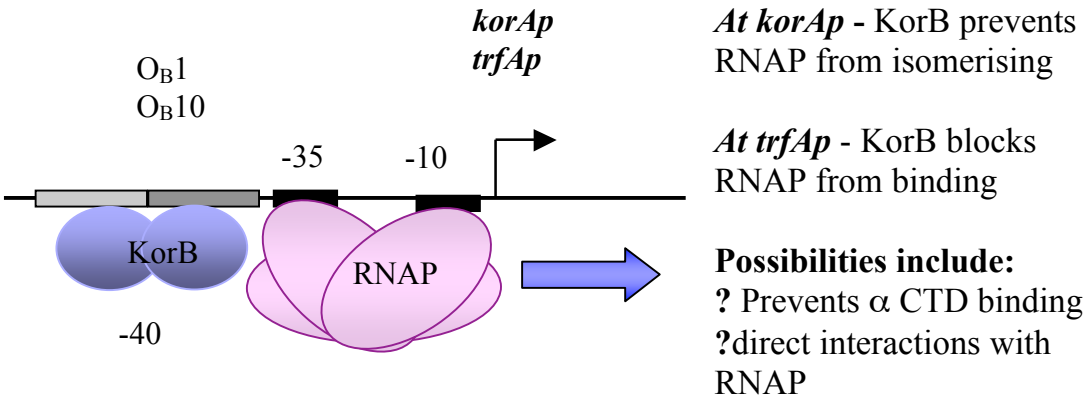


Figure 1.17: Proximal Repression by KorB (when operator sites localized approximately 40 bp upstream of the transcription start point (*tsp*). Class I promoters**:** ***trfAp, korAp*** and ***klaAp***

spreads along DNA and makes promoters inaccessible to RNAP (Radionove et al., 1999; Brier and Grossman, 2007).However, in such cases KorB is present at a considerably lower level compared to the ParB of P1 prophage i.e. 7000 dimers of ParB in an *E. coli* cell carrying P1 prophage as compared to 500 dimers of KorB in cells having RK2. KorB represses through class II operators even when the distance between operator and promoter is increased up to 1.6 kb with a little reduction in repression but no difference on cooperativity with TrbA (Bingle et al., 2005). In order to repress by looping DNA, distant binding proteins need to occur on mutually compatible faces of the DNA double helix (Richmond and Davey, 2003). KorB is a very unusual repressor protein as it can repress at a distance even when its location is rotated by 180 degrees (Bingle et al., 2005). It shows that KorB might repress at a distance by polymerising along DNA to reach RNAP or make a loop of DNA to form KorB-RNAP contact possible.

1.3.4 KorB structure

KorB structural and functional relationship has been explained previously (see **Figure 1.5 part c).** The structures of KorB central DNA binding domain and C-terminal dimerisation domain have been recently studied separately **(Figure 1.18)**. KorB has not yet been crystallised as a whole. The C-terminal dimerisation domain (Delbruck et al., 2002) consists of 5 anti-parallel β-barrel structures, which is similar to the SH3 fold present in many eukaryotic signal transduction proteins (Musacchio et al., 1994). The dimer interphase is mostly hydrophobic with leucine residues in a zipper like arrangement to promote dimerisation. At the N-terminus of the dimerisation domain is a flexible linker, connecting the central domain to the C-terminus of the protein. KorB C-terminus is also involved in DNA binding affinity and oligomerisation as both of these activities were affected by deletion of only 17 amino acids from the C-terminus (Jagura-Burdzy et al., 1999a). This shows that KorB might need to be dimeric in order to bind palindromic sequence of O_B on DNA. The C-

terminal domain also has a role in interaction with other regulatory proteins i.e. IncC, KorA, TrbA, KfrA etc. (Kostelidou et al., 1999; Jagura-Burdzy et al., 1999a; Kostelidou and Thomas, 2000; Rosche et al., 2000). The crystal structure of KorB central DNA binding domain suggested that the specificity in DNA binding is achieved by two residues outside the HTH motif, Thr211 and Arg240, which contact the inner G-C base pairs of the O_B site. Each monomer of KorB binds to a half site of O_B, wrapping around the DNA, along the trajectory of the major groove (Khare et al., 2004) whereas previous footprinting results have shown that full length KorB protects the entire O_B sequence (Balzer et al., 1992; Williams et al.,1993). Each KorB central DNA binding domain consists of eight helices. Gly 168 and Gly179 (conserved in all KorB homologue) are very important in creating sharp turns and special backbone conformation between helices 2 and 3, and helices 3 and 4 (Khare et al., 2004). KorB is an unusual DNA binding protein with net negative charge (pI=4.6) (Balzer et al., 1992). The N-terminus of the KorB central DNA binding domain consist of helices 3 and 4 and has HTH motif whereas the C-terminus consist of a four helix bundle domain. KorB monomers bound to each half clamp the operator, which is a clamshell-like structure which boosts the KorB DNA binding through additional contacts between KorB and DNA.

1.3.5 Cooperative interaction of KorB with other proteins

KorB works cooperatively by interaction with other proteins encoded by RK2 plasmid. A direct interaction has been reported between KorB and IncC *in vivo* using the yeast-two hybrid system and *in vitro* by using partially purified proteins (Rosche et al., 2000). Using yeast-two hybrid system, a 45 amino acid segment from I174 to T218 in the KorB sequence was identified to be interacting with IncC (Lukaszewicz et al., 2002). The structure of C-terminal domain of KorB shows a fold that resembles the Src-homology 3 (SH3) domain, which is well known for proteins involved in signal transduction (Delbruck et al., 2002).

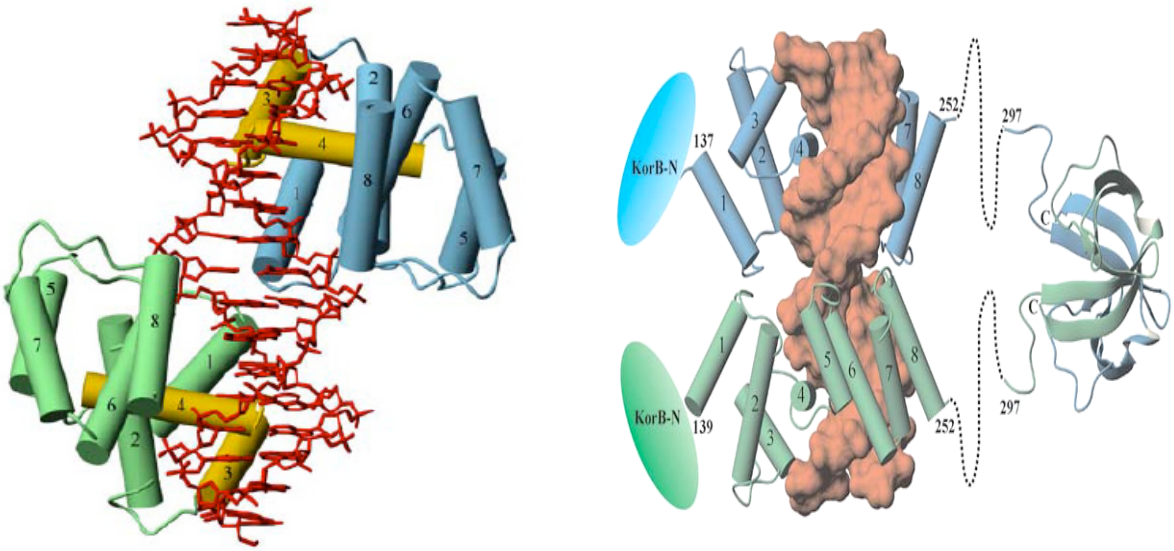


Figure 1.18: Molecular structure of KorB-O–operator complex. Two KorB-O monomers (blue and green) are occupying the half sites of O_B. The α-helices are illustrated as cylinders. Helices 3 and 4, drawn in yellow, form the HTH binding motif (Khare et al., 2004)

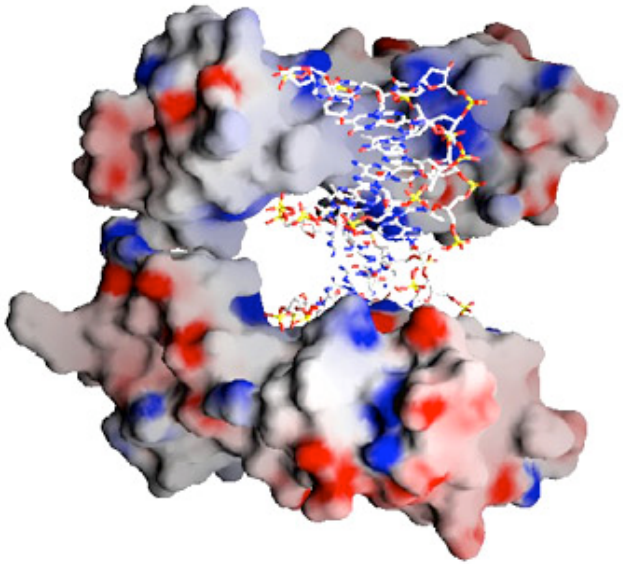


Figure 1.19: Electrostatic surface potential of KorB DNA binding domain (DBD) bound to O_B. KorB DBD binds in the major groove of the DNA, with each monomer contacting the half-site on the O_B fragment. Blue color marks positive surface and red color indicates negative charge of the protein (Khare et al., 2004).

KorA is a DNA binding protein. It exists as a homodimer in solution (Jagura-Burdzy and Thomas, 1995). Its monomer consists of 101 aa. It has a molecular mass of 11,305 Da, a predicted *pI* value of 10.38 and a net charge of +4 (Pansegrau et al., 1994). KorA can be divided into three domains based on its functions: the region I has N-terminus and is involved in dimerization (Kostelidou et al., 1998), the region II contains helix turn helix motif responsible for KorA binding to O_A operators (5´-GTTTAGCTAAAC-3´) (Smith and Thomas, 1984), the region III contains the C-terminal domain that is required for interaction with KorB (Kostelidou et al., 1999) and also in the dimerization of protein (Bhattacharayya and Figurki, 2001). A linker of 4 aa sequence separates region I and II from the C-terminal domain and might also be involved in the physical demarcation in the KorA tertiary structure (Kostelidou et al., 1999). KorA binds to seven operators on the RK2 genome and represses seven promoters nearby (*korAp, kfrAp, klaAp, kleAp, klcAp, trfAp* and *kleCp*) (Shingler et al., 1984; Smith et al., 1984; Young et al., 1985, 1987; Thomas et al., 1990). KorA homologues have recently been recorded on other plasmids that are unrelated to IncP-1:, pSB102 (Schneiker et al., 2001), pM3 (Greated et al., 2000), Pra2 (Kwong et al., 2000), pIP02 Pxf51 (Gene bank accession number NC002490).

TrbA protein, another regulator of RK2, is encoded by *trbA* preceding the *trb* operon which encodes most of the genes required for conjugative transfer (Lessl et al., 1993). Deletion analysis of TrbA showed that the C-terminal domain, which has a high degree of sequence conservation (overall 76 % similarity) with the C-terminal domain of KorA, is required for cooperativity with KorB (Zatyka et al., 2001). Proposed TrbA consensus binding sequence O_T, CNGTATATC, (Pansegrau et al., 1996) occurs at 6 sites around the RK2 genome and 5 of these sites occur at transfer gene promoters **(Figure 1.20)**. TrbA binding site (O_T : 5´-

Forward	**CNGTATATC**		
TAAACACTTT	CGGTATATCG	TTTGCCTGTG	***trbBp***
CCAACATAAT	CAGTATATCG	TGCATGCTTC	***traG1p***
GTGCATGCTT	CGGTATATCG	AAGCCGTTTA	***traG2p***
TGGCAAAATC	CTGTATATCG	TGCGAAAAAG	***traJ1p/traJ2p***
Reverse	**GATATACNG**		
GATAAGAAAA	CGATATACCG	ACGGTCGGGA	**Tn*1***
GAAAAAGGAT	GGATATACCG	AAAAAATCGC	***traJ2p/traKp***

Figure 1.20: TrbA operator binding site (O_T) sequence at different

CNGTATATC-3′) is very different from KorA binding site (O_A: 5′- GTTTAGCTAAAC-3′). All transfer gene promoters have been shown to be TrbA – regulated. TrbA binding site at *trbBp* has been mapped (Zatyka et al, 2001).

1.4 Regulation of transcription in bacteria

Repressor and activator proteins regulate all stages in bacteria starting from gene transcription initiation to degradation of proteins. Regulatory proteins bind DNA to perform these functions. Some regulatory proteins can act as repressor or activators whereas others can function as either according to the target promoter (Pérez-Rueda and Collado-vides, 2000).

1.4.1 Role of RNAP in transcription initiation

The central component in transcription regulation in bacteria is RNA polymerase (RNAP). It is a 500 kDa, multi-subunit enzyme responsible for all transcription. Core RNAP from *E. coli*, which is the best studied functionally enzyme of this class, is competent for transcription, but not for promoter-directed transcription initiation. It contains five polypeptides: β′ (1407 amino acids), β (1342 amino acids), a dimer of α (329 amino acids) and the ω subunit (91 amino acids) (Borukhov et al., 2002). RNAP core adopts a crab-claw structure as shown by the structural studies (Zhang et al., 1999; Fu et al., 1999). The β′ and β subunits form the active site of the RNAP that helps to recruit it on both templates of DNA and the RNA product (Korzheva et al., 2000). The larger amino terminal domain (αNTD; residue 1-235) dimerizes and assembles β′ and β subunits as well. The smaller carboxyl-terminal domain (αCTD; residue 250-329) is responsible for DNA binding and also has an important role at certain promoters (Gourse et al., 2000). The small ω subunit (91 amino acids) has no direct

role in transcription, but might have a role in assisting the folding of the β′ (Hampsey et al., 2001).

In bacterial cells, the core has to interact with another essential subunit, σ, and the complex is referred as the holoenzyme. The α subunit has three main functions. It helps to recognize the specific promoter sequences and then positions the RNAP holoenzyme at the target promoter. Later on, it unwinds the DNA duplex near the transcription start point (Gross et al., 1998; Wösten et al., 1998).

1.4.2 Promoter elements

Promoters are specific sequences in DNA, which control the transcription of genes. Four different DNA elements have been identified that are responsible for RNAP binding to the promoter. The two principal elements are –10 and –35 hexamer, which are located 10 and 35 base pairs (bp) upstream from the *tsp*, respectively. Promoter -10 elements are recognized by domain 2 of RNAP σ subunit (specifically region 2.4). Similarly, promoter -35 elements are recognized by domain 2 of RNAP σ subunit (specifically region 2.4). (Campbell et al., 2002; Murakami et al., 2002; Murakami et al., 2002a; Murakami et al., 2002b). Consensus hexamer sequences of -10 and -35 elements have been established and models have been generated with the help of crystallography to explain how such elements are recognized by RNAP. The other two important elements are the extended -10 element and the UP element. The extended -10 element is a 3-4 bp motif located immediately upstream of the -10 hexamer that is recognized by domain 3 of the RNAP σ subunit (Murakami et al., 2002b; Bown et al., 1997; Sanderson et al., 2003), and the UP element is a ~20 bp sequence located upstream of the promoter -35 hexamer that is recognized by the C-terminal domains of the RNAP σ subunits

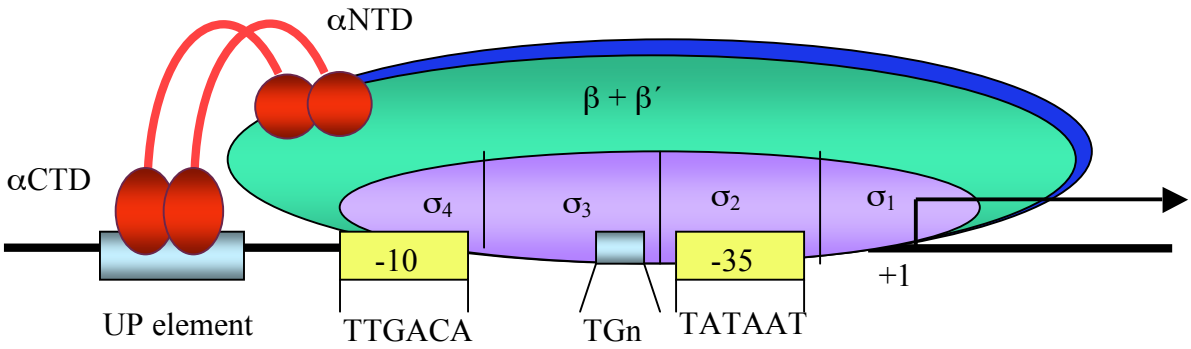


Figure 1.21: RNAP complex with the promoter- showing interaction between promoter elements and the RNAP. Model is based on crystallographic structure of the initial docking of RNAP holoenzyme to the promoter (Murakami et al., 2002b). The DNA strands and the promoter is shown in black, with the -10 and -35 elements highlighted in yellow and the TGn extended -10 and the UP elements highlighted in grayish blue. RNAP is shown with the β and β´ subunits coloured green and blue, respectively, αNTDs and αCTDs are coloured red and the different domains of σ are coloured purple. The consensus sequences for the -35(TTGACA), extended -10(TGn) and -10(TATAAT) elements are shown. (Adapted from Browning and Busby, 2004)

(Ross et al., 2001) **(Figure 1.21)**. No promoter has perfect elements, as such a promoter would bind RNAP too tightly which is not the case normally.

1.4.3 Role of promoters in transcription initiation

During transcription initiation, the RNAP binds loosely to the promoter forming a closed binary complex. The RNAP binding to DNA includes an "anchoring" of the enzyme on the –35 sequence. The first closed complex is formed, followed by an intermediate form, when RNAP interacts with the –10 region. At this stage DNA remains entirely in double-helical form. The next step involves melting of 10 - 15 bp region at the transcription start point. This leads to formation of the open complex in which the duplex DNA around the *tsp* (transcription start point) is unwound **(Figure 1.22)** (de Haseth et al., 1998; Rojo, 2001). As a next step of transcription initiation the first nucleotide is incorporated and initiation complex is formed. Eventually, when 7 – 12 nucleotides of RNA are synthesized, transition to a stable ternary complex (RNA-DNA-RNA chain) occurs by the dissociation of the sigma factor. When this happens, the specific interactions with promoters are lost and RNAP leaves as an elongation complex.

Promoters with consensus or near-consensus sequences are transcribed more efficiently. Many of the strongest bacterial promoters have UP elements and function by binding to the αCTDs of RNAP (Gourse et al., 2000). Differences in promoter sequences allow control of a wide range of promoter activities (Browning and Busby, 2004).

1.4.4 Sigma factors

RNAP regulate different promoters by using different sigma factors. Sequence comparisons revealed two unrelated families of bacterial sigma factors i.e. σ^{70} and σ^{54}. These proteins have up to four multiple domains joined together by linkers (Borukhov et al., 2002). Domains 2, 3 and 4 are involved in promoter recognition. However, function of the domain 1 is not known, and it is absent from many σ factors. Growth related and housekeeping genes, which are expressed in the exponential phase of the cell growth, are transcribed using the holoenzyme, which includes the product of the *rpoD* gene - σ^{70}. On the other hand, for transcription of some stationary phase genes, a holoenzyme containing σ^{S} is necessary. In other environmental conditions such as stress response, genes are transcribed by RNAP core combined with the alternative minor sigma subunits (Maeda et al., 2000). Both the housekeeping and alternative sigma factors are highly homologous at amino acid level and their conserved regions share similar functions (Lonetto et al., 1992; Malhotra et al., 1996).

The second, smaller family of sigma factors is the σ^{54} family. The σ^{54} recognition sequence includes short elements at positions –12 and –24. Members of this family form RNAP holoenzymes, which recognize promoters but require additional factors and a source of energy (ATP or GTP hydrolysis) for formation of transcriptionally competent promoter complexes (Buck et al., 2000).

1.4.5 Anti-Sigma factors

The global regulatory pathways are influenced by competition of alternate sigma factors for binding with the RNAP core (Farewell et al., 1998). An anti-sigma factor has the ability to inhibit a specific σ factor. Inhibiting only the σ factor that is required to recognize a particular promoter allows the anti-sigma factor to prevent transcription from the given set of promoters

(Hughes and Mathee, 1998; Helmann, 1999). The mechanisms of anti-sigma factor action vary. For example they can bind to a particular free form of sigma factor in a way that prevents its binding to the core of the holoenzyme as proposed for the Rsd protein of *E. coli* (Chadsey et al., 1998; Jishage and Ishihama, 1998). On the other side they can interact with already core-bound sigma inhibiting RNAP binding to the promoter like AsiA of the T4 prophage (Severinova et al., 1998). However the activation of a promoter by RNAP can be inhibited in other ways as well.

1.4.6 Repression of transcription initiation

Repression of transcription initiation in bacteria occurs in different ways depending on the nature of regulators controlling it. Repression of transcription can occur at different steps of the initiation process:

(1) Hindrance of RNAP binding to the promoter

(2) Melting of the promoter.

(3) Clearance of the promoter.

These methods of repression of transcription are described in the following sections.

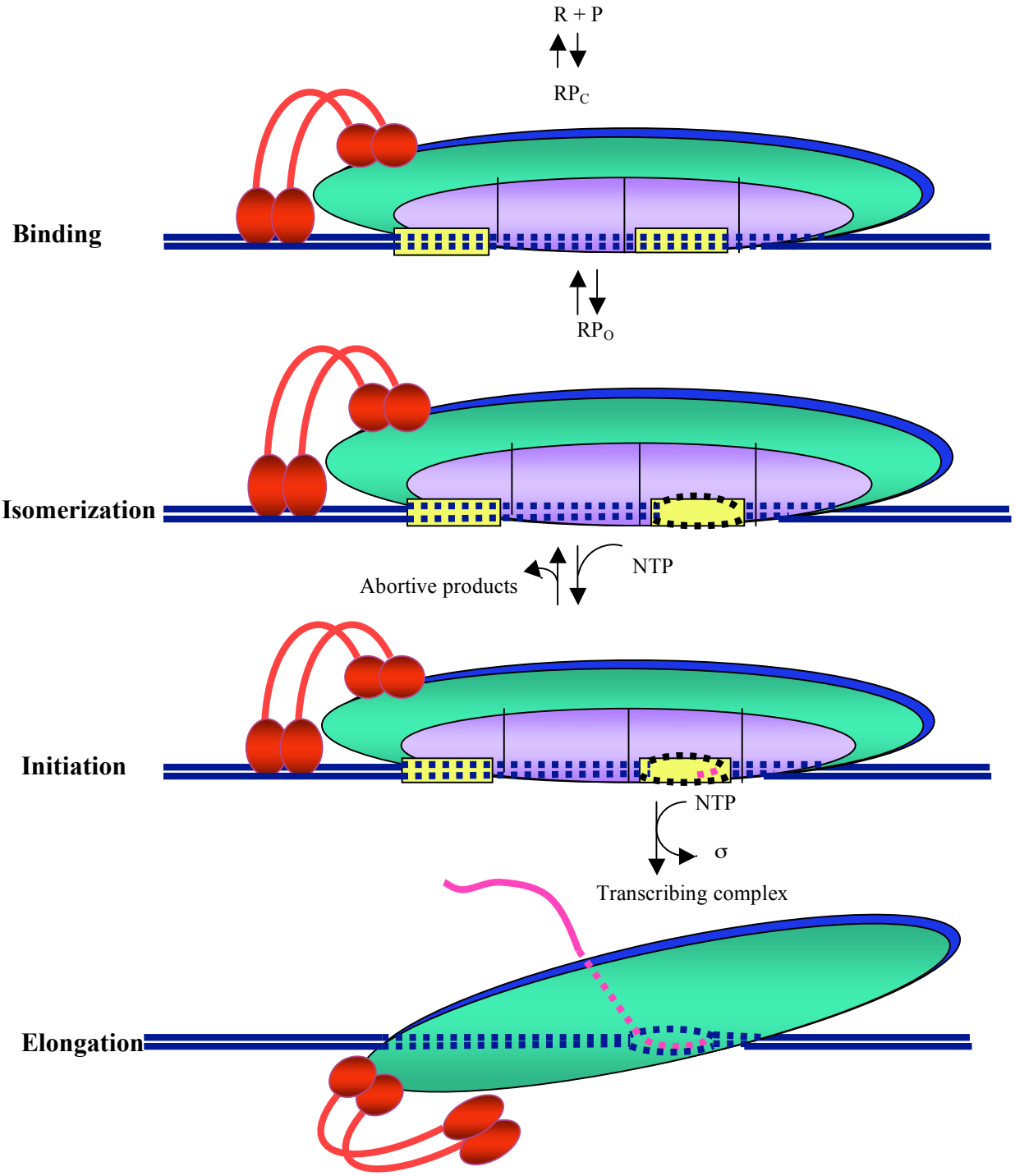


Figure 1.22: The mechanism of transcription initiation in bacteria**.** The RNAP (P) interacts with the promoter (P) to form the closed complex (RPc). The part of DNA to which RNAP is bound is shown in dashed lines. Closed complex is converted into open complex (RPo) once the DNA duplex is unwound (shown by a bubble in DNA) around the *tsp* (transcription start point). This leads to the formation of initiation complex (RP_{INT}) and the synthesis of the DNA-template-directed RNA chain (shown as a pink dashed line) begins with the phosphodiester bond between the initiating and adjacent phosphodiester nucleoside triphosphates (NTPs). Elongation is the final stage and the RNA chain length increases, shown as a solid pink line. (Adapted from Browning and Busby, 2004)

1.4.6.1 The hindrance of RNAP binding to the promoter

One of the most common and simple ways to repress transcription initiation is the hindrance of RNAP binding to the promoter. It can be due to the action of several anti-sigma factors as described above. In this type of repression, regulator (repressor) binding site is present either close to, overlapping, or at the promoter where RNAP binds and forms an open complex. There are several repressors that prevent transcription initiation in this way.

One example of such repression is a repressor of λ phage, the cI protein. It binds to the specific operator sequences O_R1, O_R2 and O_R3. The O_R1 and O_R2 sequences overlap the divergently orientated promoters P_R and P_{RM} (Hawley et al., 1985). O_R3 is an intrinsically weaker binding site and is filled only if repressor concentration increases to a certain higher level that would result in the full occupancy of O_R1 and O_R2. Cooperative binding of the cI protein to the operator sequences excludes RNAP from the P_R that transcribes genes required for the lytic cycle. At the same time RNAP also binds to the P_{RM}, from which transcription of the repressor gene occurs and which is responsible for maintenance of the lysogenic cycle **(Figure 1.23**). In fact binding of cI to O_R1 and O_R2 activates P_{RM}. When bound to site O_R3, cI shuts off further expression of the repressor gene by excluding RNAP from P_{RM}.

The p4 protein of the Φ29 phage represses the early A2b promoter by binding upstream of the RNAP (Monsalve et al., 1997). Similar mechanism has been described for the repression of:

- the *uvrA* promoter, where binding of the repressor protein occludes RNAP (Bertrand-Burggraf et al., 1987)
- the *gyrA* promoter by the Fis (factor of inversion stimulation) whose binding site overlaps –10 and –35 elements (Schneider et al., 1999)

- the *lac* promoter by LacR repressor binding to the O_1 operator, which overlaps the *lac* promoter and inhibits the initiation of transcription of the *lac* operon (Schlax et al., 1995) **(Figure 1.24)**.

The mechanism of sigma factors antagonism is another way of inhibiting the RNAP binding to the promoter. When sequences of two promoters overlap, RNAP bound to one of them can repress binding of the other molecule to the second promoter. The RNAP holoenzyme complex with sigma factors σ^{54} (σ^{N}) is an example of such an activity. The σ^{54} holoenzyme forms a closed complex and occupies the promoter. This closed complex is unusually stable and cannot isomerize to open complex without the help of a transcriptional activator (Buck et al., 2000). The mechanism of sigma factors antagonism was described for *Pseudomonas aeruginosa* gene *algD*, which can be expressed from two overlapping promoters recognized by two holoenzymes – σ^{54}-RNAP or σ^{E}-RNAP. The σ^{54}-RNAP creates a very stable closed complex on the σ^{54}-dependent promoter and blocks access of σ^{E}-RNAP to its promoter sites. Expression of the σ^{E}-dependent promoter is impossible without activation, when the σ^{54}-RNAP is bound (Boucher et al., 2000).

1.4.6.2 Inhibition of an activator

In this type of regulation, a repressor protein also acts as an anti-activator. The inhibition of an activator (or anti-activation) has been described for the regulation of those promoters that are controlled by cAMP receptor protein (CRP) for their activity, but are repressed by the cytidine controlled repressor (CytR). In the absence of cytidine, CytR binds to DNA between two CRP dimers and then interacts with DNA and CRP dimers. This is how CytR completely prevents CRP from activating the transcription. CytR is a specific antagonist of CRP-

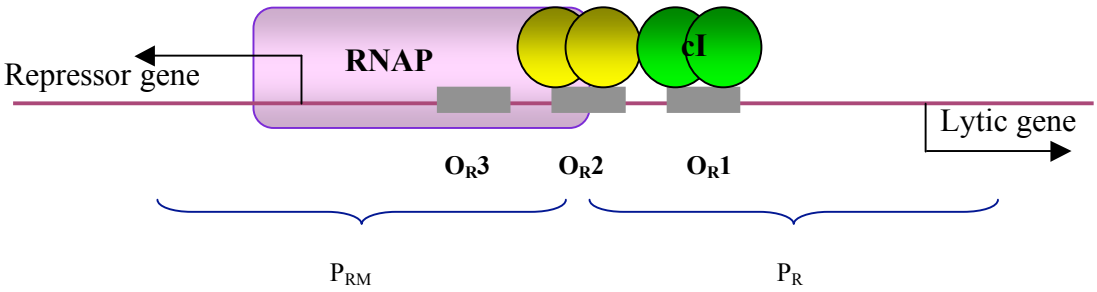


Figure 1. 23: Genetic organization of the O_R region of λ phage. The grey boxes indicate cI binding sites; the arrows show transcription start point for repressor and lytic genes (Monsalve et al., 1997).

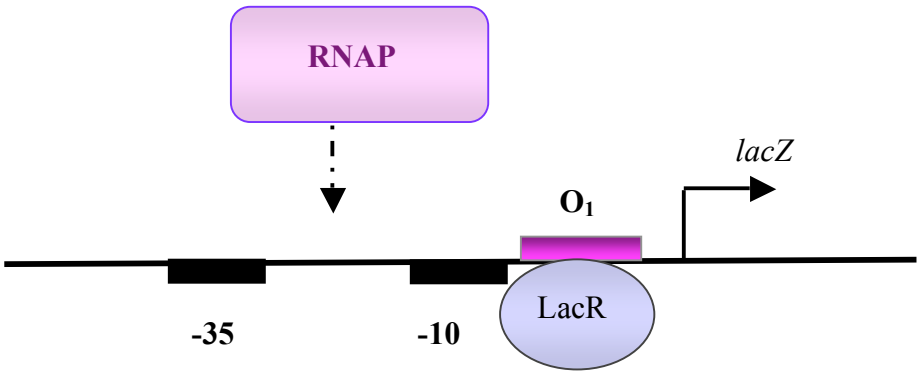


Figure 1.24: Genetic organization of the *lacZ* promoter region. A blue box indicates LacR binding site; the black boxes indicate promoter sequences, an arrow shows the start point of transcription of *lacZ* gene; the numbers show position of the promoter hexamers relative to the transcription start point.

dependant initiations. For example the *deo*P2 promoter of *E. coli* is repressed by CytR protein, which binds 70 bp upstream of the transcription start point between two CRP dimers that bind to the positions –40 and –93 (Perini et al., 1996; Shin et al., 2001).

Repression of transcription initiation can also result from distant binding repressors. Such a repression is called distal repression. Distal repressors play a great role in genome-wide expression. Promoters that are controlled by distal repressors require activation in order to form the open complex. At such promoters repressors bind to the sites between the promoters and activator binding sites, and result in the bending of DNA, which causes interruption of the contact between the RNAP and activator. This leads to inhibition of promoter melting. One of the examples of such a mechanism is levanase operon of *Bacillus subtilis*, which is regulated by the LevR activator protein that interacts with upstream activating sequences (UAS) and stimulates transcription of the RNAP complex bound at the promoter. The CcpA protein binds to the catabolite responsive elements (CRE) that are localised in the promoter region between LevR target UAS and the –12 and –24 of the promoter. This binding of CcpA in the promoter region may modify the condition of the protein – protein interaction between LevR and RNAP associated with σ^{54} that is essential for the melting of DNA and the activation of transcription (Martin-Verstraete et al., 1995).

Distal repression via DNA loop formation has also been reported for GalR protein of the *gal* operon in *E. coli.* GalR binds (as a dimer) to two operator elements, O_E and O_1, which encompass a 113 bp DNA segment comprising the *gal* promoters. Interaction between dimers of GalR bound on DNA results in the formation of loop, which also requires the architectural protein HU (Semsey et al., 2002).

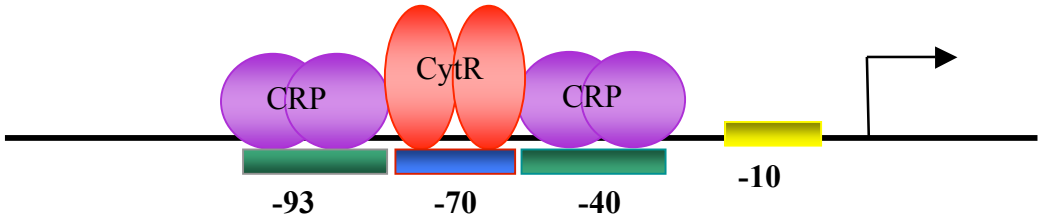


Figure 1.25: Regulatory structure of the *deo*P2 promoter. The yellow box indicates the –10 region of the promoter; the green boxes indicate CRP binding sites; the blue box indicate CytR binding site; an arrow shows the start point of transcription; the numbers show the position of the protein binding sites and the promoter hexamer relative to the transcription start point. (Shin et al. 2001).

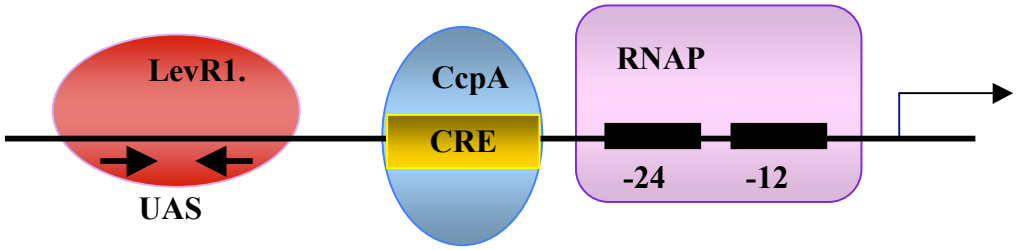


Figure 1.26: Model of repression of *levanase* operon. The black boxes indicate the promoter sequences; the yellow box indicates the catabolite responsive element (CRE) – binding site of the CcpA protein; two thick arrows arranged face-to-face indicate the target for the LevR protein – upstream activating sequences (UAS); the single arrow shows the start point of the transcription of the *levanase* operon; the numbers show the position of the promoter sequences relative to the transcription start point (Martin-Verstraete et al. 1995).

Another example of anti-activation mechanism is the *E. coli nir* promoter. Repressor proteins Fis (-142 and +23), IHF (integration host factor) (-88) and H-NS (histone-like nucleoid-structuring protein) bind to the upstream sequences of the promoter and repress transcription of that promoter. Binding and interaction of these three proteins results in the formation at the promoter of a highly ordered nucleoprotein structure that represses FNR-dependent activation (Browning et al., 2000).

1.4.6.3 Inhibition of open complex formation

In this type of repression, repressors do not prevent simultaneous binding of RNAP but do not allow completion of the initiation of transcription. These repressors have their binding site partially or totally overlapping RNAP binding sequences. Transcription of the P_{ant} and P_{mnt} promoters in the *immunity I* operon is repressed by the Arc protein of the bacteriophage P22 (Susskind and Youderian, 1983; Vershon et al., 1987). The Arc protein binds to the tandem sites of the *arc* operators, which are placed between the –10 and –35 elements and thus blocks the isomerisation of DNA-RNAP complex from closed to open (Vershon et al., 1987).

The mercury resistance (*mer*) operon encodes the *merR* gene for the metal-responsive regulatory protein MerR. MerR regulates its own transcription as well as transcription of the divergently transcribed structural genes. MerR binding sites are present between the –10 and –35 elements of the *merTPCAD* promoter, to which it binds and causes repression. In the absence of mercury, MerR retards isomerisation to open complex (Summers, 1992)

In other cases the operator binding sites of regulatory proteins (such as KorB of RK2 plasmid) do not overlap the elements recognized by RNAP. However, transition from closed to open complex is still inhibited. Experiments have shown that KorB (global regulator of RK2)

allows simultaneous binding of RNAP to the *korABF* promoter but blocks transition from closed to open complex by melting the –10 region (Williams et al., 1993).

1.4.6.4 Inhibition of promoter clearance

Some of the regulator proteins repress transcription initiation by preventing RNAP from escaping from the promoter. The regulatory protein p4 of *Bacillus subtilis* phage Φ29 represses transcription from early viral promoter A2c. The p4 repressor binds upstream of the sequence recognised by RNAP and interacts with the C-terminal domain of the RNAP α subunit. The interaction between α subunit of RNAP and p4 anchors RNAP at the promoter in such a way that an initial transcribing complex can make a short abortive transcript but cannot clear the promoter **(Figure 1.27)**. The stabilization of RNAP at the A2c promoter leads to repression (Monsalve et al., 1997).

LacI is a repressor protein that allows simultaneous binding of RNAP but interferes with subsequent steps in initiation and transcript extension beyond +4. The LacI repressor binds immediately downstream of the promoter at position located at +13 and +15 and inhibits the T7 phage RNAP (Lopez et al., 1998). It was shown for some promoters that insertion of phased A-(adenine) tracts in the upstream region inhibits their activity by blocking escape of RNAP from the promoter (Ellinger et al., 1994).

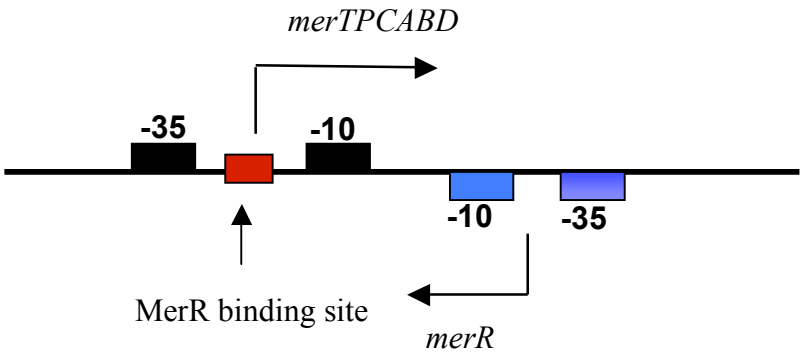


Figure 1.27: General structure of the *mer* operon**.** The black boxes with numbers indicate the promoter sequences of the *merTPCABDp* and the single black box symbolises the *mer* operon; the blue boxes with numbers below indicate the promoter sequences of the *merR* gene; the red box indicates the MerR binding site; the arrows show transcription start point of the *merTPCABD* and *merR*; the numbers show the positions of the promoter hexamers relative to the transcription start points of their genes. After Summers (1992).

1.4.7 Influence of DNA topology on regulation

Interaction of regulators (i.e. repressors and activators) with RNAP and the promoter is controlled by the DNA topology (i.e. unwinding, bending and looping). One of the examples includes MerR. It mediates unwinding of DNA, which leads to activation of the *merT* promoter. MerR in complex with mercury binds to the operators between –10 and –35 elements and underwinds the 19 bp spacer. That action leads to a realignment of promoter and allows RNAP to bind (Ansari et al., 1995) **(Figure 1.28)**.

The C protein of the bacteriophage Mu binds to the operator sites placed from –28 to –57, which partially overlaps the –35 of the *mom* promoter. The binding of MuC mediates asymmetric distortion and unwinding of DNA. These changes lead to reorientation of the –10 and –35 regions of the *mom* promoter, which allows RNAP to bind to an otherwise inaccessible promoter (Basak and Nagaraja, 1998) **(Figure 1.29)**.

Upstream phased A-tract sequences bend DNA and modulate transcription substantially. A-tracts mediate repression by blocking RNAP clearance from the promoter (Ellinger et al., 1994). The promoters, which were rate-limited at different steps of transcription initiation, respond differently to A-tracts. A promoter, which was limited at the formation of the open complex, was strengthened by A-tracts, probably by facilitating the initial binding of RNAP. On the other side a promoter which was rate-limited at the following step of the transcription initiation was inhibited by A-tracts, possibly by reducing ability of RNAP to escape from elongation complex (Ellinger et al., 1994).

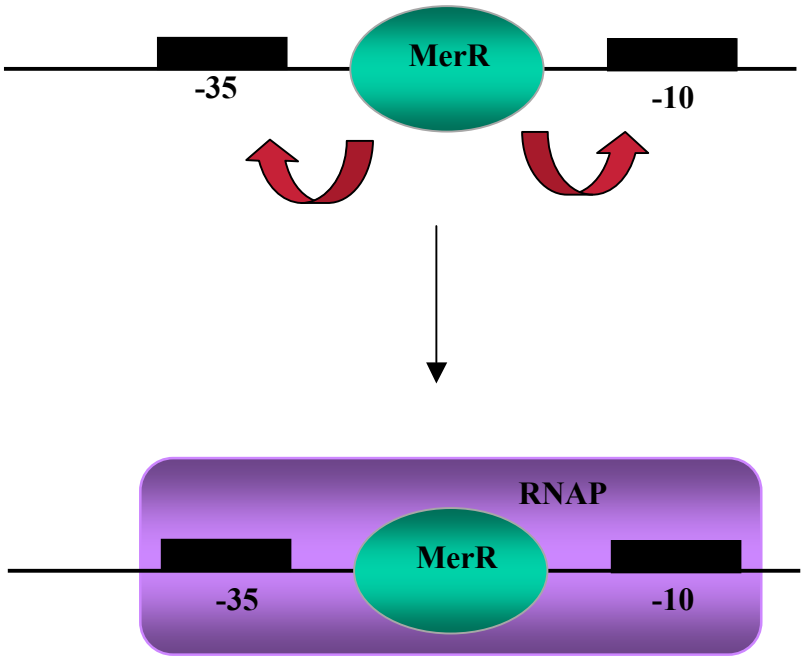


Figure 1.28: The regulatory region of *merTp*. Binding of the MerR protein at *merT* promoter leads to the repositioning for RNAP occupancy. The black boxes indicate sequences of the promoter; the numbers show position of the promoter hexamers relative to the transcription start point; the red arrows symbolise the underwinding of the DNA leading to the reorientation of the promoter sequences. Adapted from Basak and Nagaraja, 1998.

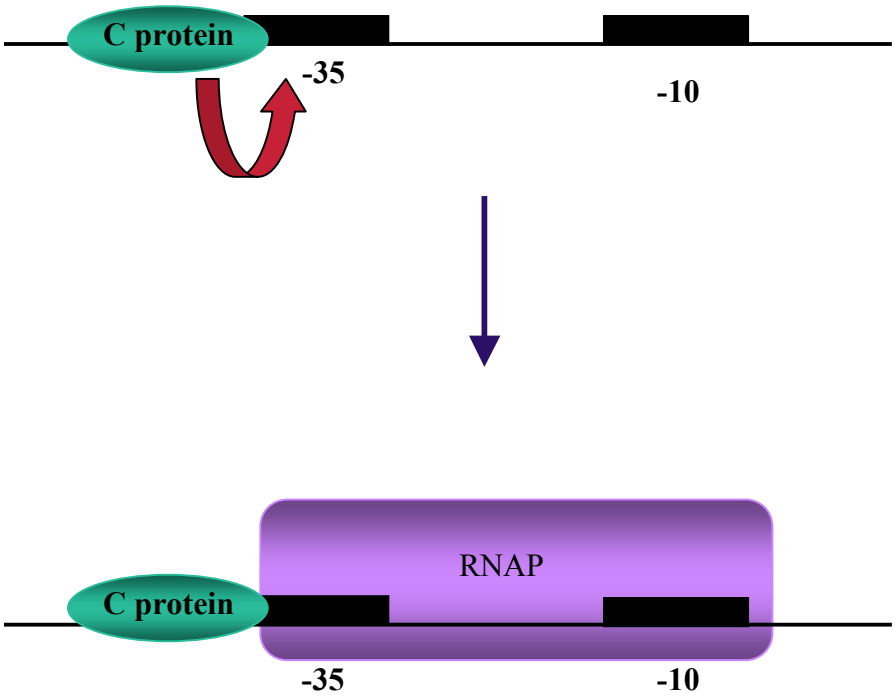


Figure 1.29: The regulatory region of *momp*. Binding of MuC at *momp* causes reorientation of the promoter for recognition and binding of RNAP. The black boxes indicate sequences of the promoter; the numbers show the position of the promoter hexamers relative to the transcription start point; a white arrow symbolises the underwinding of the DNA leading to the reorientation of the promoter sequences. Adapted from Basak and Nagaraja, 1998.

The mechanism of activation of the *ilv*P_G promoter, which is described in detail in next section, depends on both structural transition of DNA and protein-induced DNA bending. The region between –67 and –153 is rich in adenines and thymines, and is destabilized in the supercoiled template. The binding of IHF to sites between –80 and –95 stabilizes the upstream (A+T)-rich region, destabilizes pairing at positions –11 and –10 and enhances the transition of RNAP from closed to open complexes (Sheridan et al., 1998; Parekh et al., 1996).

The gal operon promoters *P*1 and *P*2 are repressed by the formation of a DNA loop. Histone-like protein HU binds to a specific locus (*hsb*), which leads to the formation of loop by interaction between the GalR protein bound at to two operators (O_E and O_I). This higher order DNA-multiprotein complex containing GalR, HU and supercoiled DNA mediates repression of *gal* operon (Lewis et al., 1999; Adhya, 2002).

DNA looping influences repression as well as activation. AraR protein of *B. subtilis* is the first member of the GntR family of bacterial regulators for which DNA looping was proposed as the mechanism of repression. In the *ara* regulon, AraR target promoters have precisely spaced duplicate operators and/or a special inter-operator sequence. The binding of two AraR molecules to properly spaced operators leads to the formation of the loop. When the loop is formed two molecules of repressor communicate, which is crucial for the control of the *ara* regulon (Mota et al., 2001).

The AraC of *E. coli* regulates transcription from the ara_{BAD} promoter. Each AraC DNA-binding domain contains two HTH motifs each of which bind to DNA, so a monomer is like a heterodimer fused to form a single polypeptide. In the absence of arabinose AraC binds to

two half-sites separated by 210 bp, forms a DNA loop, and causes repression **(Figure 1.31)**. On the addition of arabinose AraC prefers to bind to adjacently located half-sites and induces transcription from the ara_{BAD} promoter (Harmer et al., 2001).

H-NS represses *virF* promoter (from *Shigella sp.*) by cooperatively binding at two regions on the supercoiled DNA. One of the H-NS binding site overlaps the RNAP binding site whereas the second is located at 250 bp upstream of the RNAP binding site (Falconi et al. 1998). The *virF* promoter fragment having two H-NS sites undergoes a specific temperature-dependant conformational transition. This modification of the DNA target may alter a cooperative interaction between H-NS molecules, which are bound at two distant sites and modulate regulation of virulence gene expression. A similar situation occurs in other H-NS-regulated promoters such as *fimE* and *fimR* recombinase genes, which control the orientation of the type 1 fimbrial phase switch (Olsen et al., 1998).

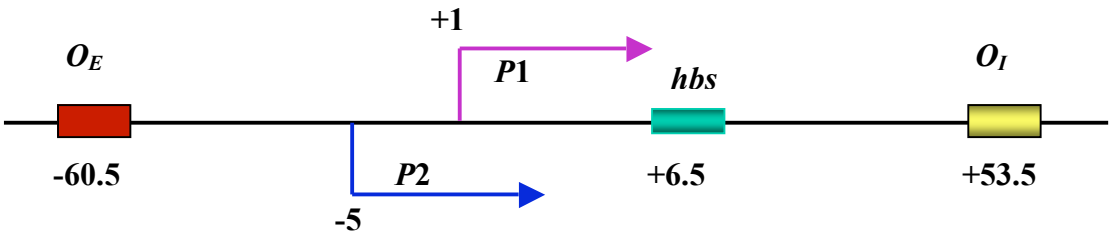


Figure 1.30: The regulatory region of the *gal* operon. O_E and O_I are operators binding sites of GalR; *hbs* is the HU binding site. The arrows indicate the start point of transcription of *P*1 and *P*2 promoters. The numbers show the position of the corresponding protein binding and transcription start sites relative to the *P*1 (+1) (Lewis et al., 2002).

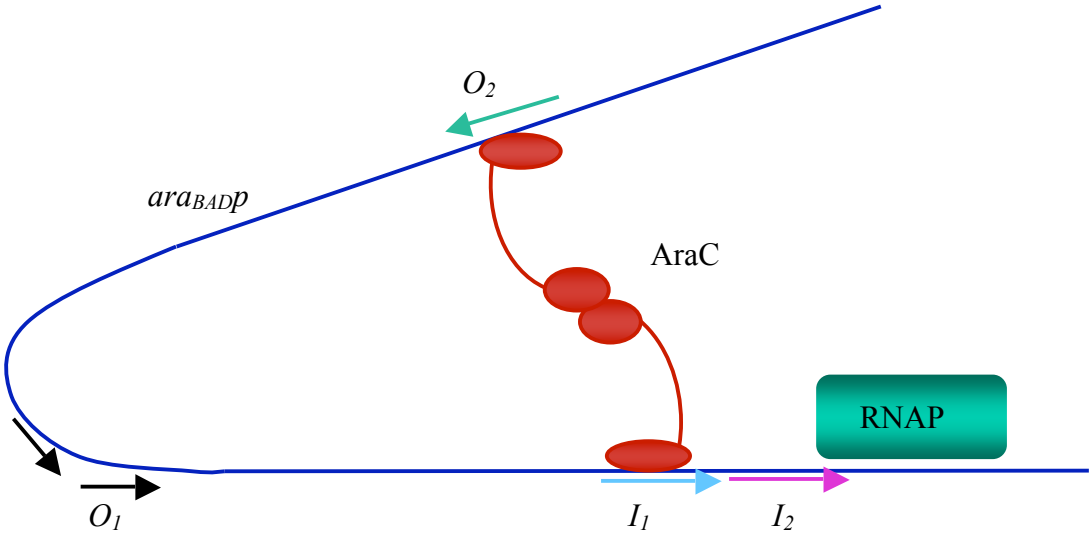


Figure 1.31: Regulatory region of the $ara_{BAD}p$. The arrows indicate AraC binding sites. In the absence of arabinose AraC binds to the sites O_2 and I_1 and causes repression of transcription from the promoter. However, in the presence of the arabinose AraC binds to two adjacent operators I_1 and I_2 and activates transcription (Harmer et al., 2001).

RK2 is a broad-host-range plasmid from incompatibility group IncP-1. RK2 gene regulation system is very unique. Its complex circuits are regulated by repressor proteins (i.e. KorB, KorA, TrbA, KorC and more which are not known yet), which cooperate with each other to exert a tight control on vital plasmid functions like replication, partitioning and transfer. KorB represses and regulate the activity of many promoters, although the mechanisms of repression by KorB are not clear yet.

1.5 The role of ParB protein as gene silencers

Transcriptional silencing is a very interesting phenomenon that occurs by binding of the regulatory proteins to *cis*-acting sites that are called silencers as they mediate silencing by bi-directional inactivation of gene expression. During gene silencing, DNA forms a structure that prevents the interaction of sequence-specific DNA-binding proteins with their cognate sequences, and thus gene expression is blocked (Rine, 1999).

The best studied examples of gene silencing include the H-NS protein that can completely shut down the expression of genes by forming extended nucleoprotein structures at the *E. coli* and *Salmonella typhimurium proU* operons and the *E. coli bgl* promoter. Such regions are located upstream or downstream of the target promoter, and thus repress transcription (Gowrishankar et al., 1996). This is how H-NS results in the low expression of the *bgl* promoter by silencing sequences located upstream and downstream of the promoter (Caramel and Schnetz, 1998).

Transcriptional silencing has also been reported for ParB and SopB partitioning proteins of P1 and F plasmids respectively. ParB and SopB bind to a *cis* site and spread along the DNA, resulting in the silencing of genes up to 10 kb from the binding site and interfering with F

plasmid replication (Lynch and Wang, 1995; Lobocka and Yarmolinsky, 1996; Hanai et al., 1996; Kim et al.,1998; Rodionov et al., 1999). Spreading of ParB proteins was discovered by a combination of its silencing of the expression of genes as well as immunoprecipitation of DNA cross-linked to ParB (Lobocka and Yarmolinsky, 1996; Rodionov et al., 1999). ParG, an analogue of ParB, is a tiny partitioning protein of the plasmid TP228 that can oligomerize when bound to DNA (Golovanov et al., 2003). Extensive spreading is not essential for the pairing of plasmids (Lobocka et al., 1996) but there is some evidence for the involvement of ParB in pairing of *cis*-acting partitioning sites (Edgar et al., 2001). Spreading of ParB proteins have supplementary effects for the partitioning process. ParB-wrapped DNA of partitioning plasmids reduces the chances of their dimerization and results in more segregating units per dividing cell (Rodionov and Yarmolinsky, 2004). This may be an additional way in which ParB plays an important role in the maintenance of plasmids by spreading along DNA. The region surrounding the *parS* site is AT rich and may permit the observed ParB spreading of up to 500 bp either side of the site, through non-specific DNA binding by the HTH (Schumacher et al., 2007; Surtees and Funnell, 1999). Spreading may also be aided by interactions between adjacent ParB N-terminal domains, which have been shown to form transient oligomers (Surtees and Funnell, 1999). ParB does not prevent all transcription events in the region between *parS* and a reporter that it represses at a distance – this suggests that ParB does not totally coat and render inert the DNA in the region that it is silencing (Lobocka and Yarmolinsky, 1996). KorB can cause gene repression up to 2 kb from its binding site and thus play a great role in controlling genome wide expression (Bingle et al., 2005). Spo0J (ParB homologue of *Bacillus subtilis*) has also been shown to spread along DNA up to 10 kb from its binding site (Breier and Grossman, 2007).

1.6 Aims and objectives of this study

The main objectives of this study are:

1. Identifying the critical residues involved in DNA binding properties of the negatively charged KorB and cooperativity with KorA and TrbA using substitution mutagenesis.
2. Ascertaining whether KorB can act as a gene silencing protein.
3. Investigating how deletion of the different regions of KorB affect its overall structure, DNA binding, repression and cooperativity with TrbA and KorA.
4. Estimating the effect of the His-tag on the N-terminus of KorB in its ability to bind DNA.
5. Studying the interaction of KorA Y84A with KorB *in vitro*, as this residue has been reported to be critical for interaction *in vivo.*

Analysis of the KorB mutants will provide a clearer understanding of the biochemical and biophysical properties of KorB, and of the mechanisms by which KorB binds DNA and represses transcription of promoters (*korAp* and *trbBp)* from proximal and distal O_B. It will also shed light on the ways RK2 tightly controls the expression of genes. It will broaden the knowledge of ParB proteins in plasmid as well as chromosomal system to which KorB is more closely related (Spo0J of *Bacillus subtilis).*

Chapter 2: Materials and Methods

2.1 Bacterial strains and growth conditions

The *E. coli* strains used in the experiments are described in **Table 2.1**.

Table 2.1: *E. coli* strains used in the experiments

Strain	Genotype	Comments	Source/ Reference
DH5α	*supE44 ΔlacU61 (ϕ80lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	A recombinant-deficient amber suppressing strain used for the purpose of constructing and growing plasmids	(Sambrook et al., 1989)
BL21	F^- *ompT*, *hsdS*$_B$ ($r_B^- m_B^-$) *gal*, *dcm* (phage DE3)	Used for over-expression of proteins from pET 28a expression vectors with the bacteriophage T7 promoter (e.g. pET). The T7 RNA polymerase is expressed from *λDE3*, which is integrated into the chromosome	(Novagen)
BL21 pLysS	F^- *ompT*, *hsdS*$_B$ ($r_B^- m_B^-$) *gal*, *dcm* (phage DE3) pLysS	Same as BL21 but contains plasmid pLyS, which expresses T7 lysozyme, a natural inhibitor of T7 RNA polymerase. This allows for additional regulation of gene expression.	(Novagen)
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Amber suppressing strain used for bioassays.	(Sambrook et al., 1989)

Bacteria were grown in Luria-Bertani (LB) broth (LB-broth) or on Luria-Bertani (LB) agar (L-agar) (Miller, 1992) at 37°C. Antibiotic resistance was used to select for resistance by addition of: benzyl penicillin sodium salt (Pn, at 150 μg/ml in liquid medium and 300 μg/ml in solid medium), kanamycin sulphate (Km, 50 μg/ml), chloramphenicol (Cm, 30 μg/ml) and streptomycine sulphate (Sm, 30 μg/ml). All medium was sterilised by autoclaving (before adding antibiotics, maltose or IPTG).

2.2. Plasmids used in this study

Plasmids used in this study are shown in the **Table 2.2**. .

Table 2.2: Plasmids used in this work

Plasmid	**Ab^R**	**Replicon**	**Important Characteristics**	**References**
pET-28a	Km^R	pMB1	Expression vector carrying terminal His-tag	New England Biolabs
pGBT331	Km^R	pMB1	pET-28a with T7-tag removed. *korB* inserted in MCS. Found to be mutated, so replacement was made.	Lukaszewicz et al., 2002
pET-28a-KorB	Km^R	pMB1	pET-28a with T7-tag removed. *korB* inserted in MCS by *EcoR*I-*Sal*I	Batt et al., unpublished
pBR322	Ap^R Tc^R	pMB1	*E.coli* cloning vector	Sambrook et al., 1989

pGEM®-T Easy	Pn^R	pMB1	T-tailed cloning vector	Promega
pACYC184	Cm^R Tc^R	p15A	Cloning vector	Rose, 1988
pPT01	Km^R	pSC101	*xylE* promoter probe	Thorsted et al. 1996
RK2	Km^R Pn^R Tc^R	IncP α	Wild type, naturally occurring, broad-host range, capable of transfer between virtually all Gram-negatives	Ingram et al., 1973
R751	Tm^R	IncP-1β	Wild type, naturally occurring , broad-host range	Thorsted et al., 1998
pGBT63	Km^R	pSC101	*trbBp-xylE*	Jagura-Burdzy et al., 1992
pLB40	Km^R	pSC101	*trbBp-xylE*, operator cloned in position -40	Bingle et al., 2005
pDM1.2	Sm^R	IncQ	Expression vector, *lacI^q-tacp*	Macartney et al., 1997
pLB25	Sm^R	IncQ	Expression vector, *lacI^q-tacp -trbA*	Bingle et al., 2005
pDM1.21	Sm^R	IncQ	WT KorB expression vector, *lacI^q-tacp. korB* has been ligated within *Eco*RI.	Macartney et al., 1997
pGBT30	Pn^R	pMB1	Expression vector, *lacI^q-tacp*	Jagura-Burdzy et al., 1991

pMMV811	Pn^R	pMB1	WT KorB expression vector, *lacI^q-tacp*	Williams et al., 1993
pMZT24	Pn^R	pMB1	WT TrbA expression vector, *lacI^q-tacp*	Williams et al., 1993
pLB125	Km^R	pSC101	*korAp* - distal O_B9 in pPT01	Bingle et al., 2005
pLB101	Km^R	pSC101	*tsp*–O_B centre distance 278 bp	Bingle et al., 2005
pLB102	Km^R	pSC101	*tsp*–O_B centre distance 366 bp	Bingle et al., 2005
pLB103	Km^R	pSC101	*tsp*–O_B centre distance 533 bp	Bingle et al., 2005
pLB104	Km^R	pSC101	*tsp*–O_B centre distance 636 bp	Bingle et al., 2005
pLB105	Km^R	pSC101	*tsp*–O_B centre distance 1563 bp	Bingle et al., 2005
pLB117	Km^R	pSC101	*trbBp-xylE* with proximal upstream O_B; O_B centre–*tsp* distance 50 bp	Bingle et al., 2005
pLB118	Km^R	pSC101	*trbBp-xylE* with proximal upstream O_B; O_T–O_B centre–centre distance 55 bp	Bingle et al., 2005
pSTM11	Sm^R	IncQ	Expression vector, *lacI^q-tacp -KorA*	This thesis.

2.3 Construction of KorB substitution mutants

These KorB substitution mutants were created using PCR. Chosen amino acids were target for site directed mutagenesis. A number of primers were designed to introduce mutations into KorB. In the middle of KorB a new restriction site (*KpnI)* was introduced. Two primers were designed, each carrying a substitution, which introduced a restriction site by changing the nucleotide sequence but not the amino acid sequence. One of the primers runs in the direction of the start codon and the second towards the stop codon of *korB.* These primers were used together with two other primers. One of them runs through the start codon and introduces *EcoRI* site at the beginning of *korB*- KorB1 5'-CC*GAATTC*ATGACTGCGGTCAAGCCAA-3'. The second runs from the stop codon and introduces a *SalI* site just after the end of KorB gene- *SalI stop* 5'-CGCTGTCT*GTCGAC*ATCAGCCCTC-3'. These set of primers allow creating two "halves" of the WT *korB* with a new restriction site **Figure 2.1**. The second set of primers was designed in order to create substitution mutants. These primers introduced the same unique restriction site and a single amino acid change (i.e. E237A, E237A K244A, E237AR240A, E237AD234A). These primers were used in PCR together with KorB1 primers to amplify "halves" of *korB* gene and a unique restriction site *KpnI* on the one end and *SalI* or *EcoRI* site on the other end were cloned into pGEMT-E vector and their sequence was determined. The halves of KorB with the correct sequence were combined in order to create a whole functional *korB* ORF. Vector DNA carrying "half" of the gene was cut using *SalI* and *KpnI* and in this was WT or mutated "half" of the gene was obtained. This fragment was subsequently cloned into the vector, cut with *Sal*I and *Kpn*I, carrying the second "half" of the gene. At this stage two WT "halves" were combined to give the whole WT *korB* ORF with *EcoR*I and *Sal*I sites on its ends or one WT "half" was combined with the "half" carrying the mutation, resulted in the creation of *korB* ORFs with single

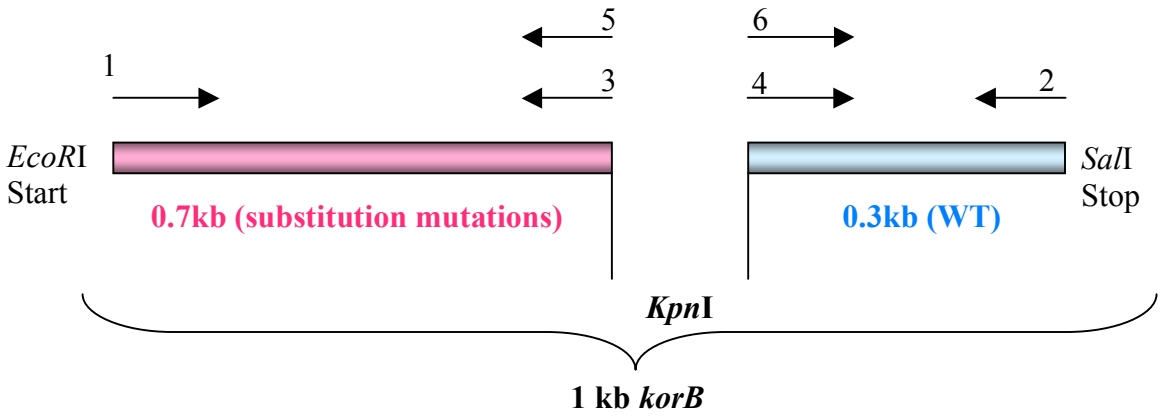


Figure 2.1: Diagram showing the construction of "halves" of *korB* and introduction of new unique *Kpn*I restriction site. The arrows symbolise primers. Pair of primers: primer1- KorB1 with primer 3 or 5 and primer 2 (*Sal*I stop) with primer 4 or 6. Primer 3 and 4 introduce a new unique restriction site. Primer 5 and 6 introduce a new restriction site and single amino acid substitution.

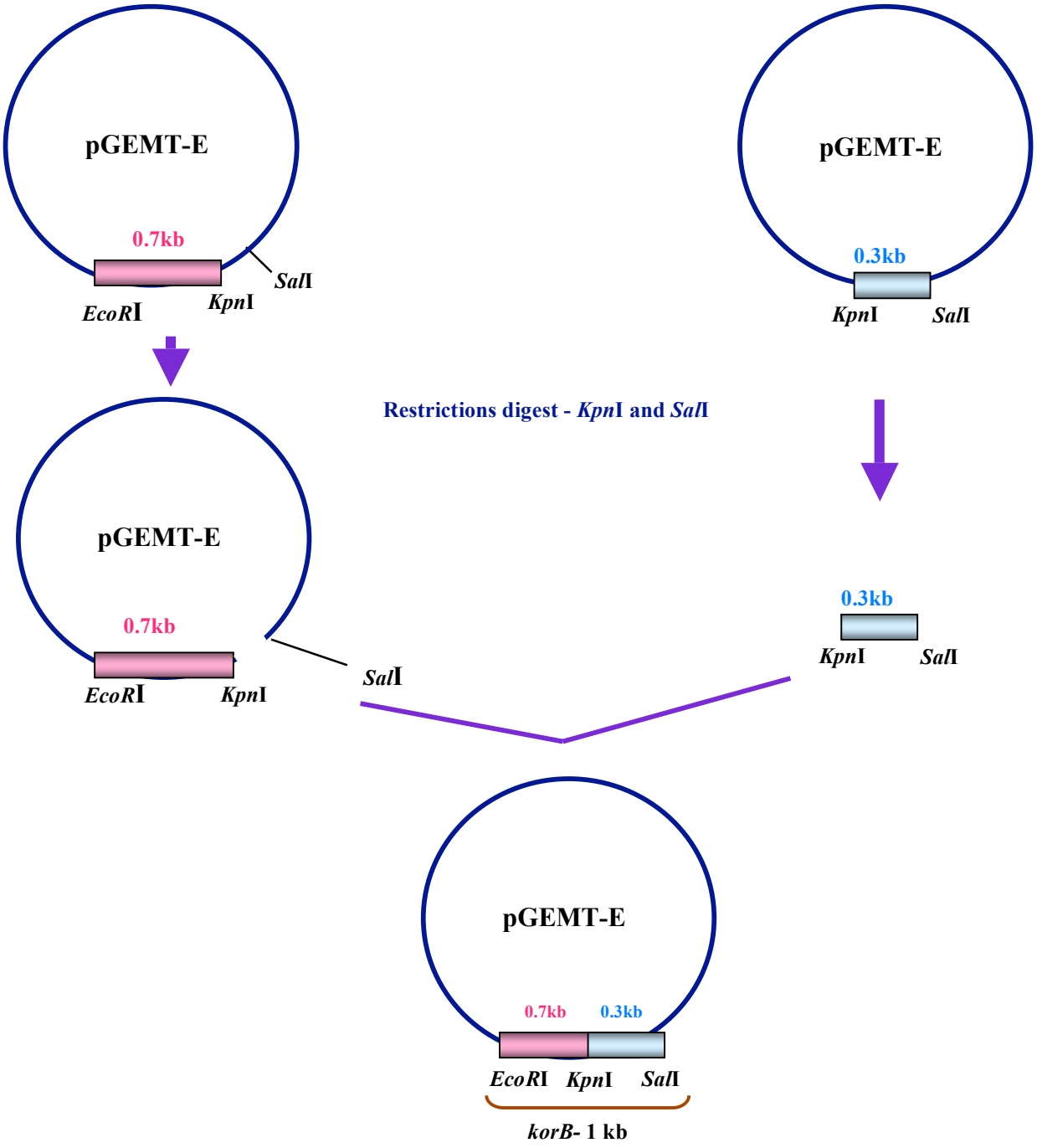


Figure 2.2: Diagram showing the construction of whole length *korB* (WTor substitution mutants) with new unique *Kpn*I restriction site. Pink and blue boxes symbolise the "halves" of *korB* created in the PCR reaction as presented in Figure 2.1, which after determination of their sequence were combined to create a full *korB* ORF.

Table 2.3: Primers used in PCR to amplify halves of the KorB (WT/substitution mutant) ORFs in pairs with KorB1 primer

KorB mutation	Primers combined with KorB1[a]
1. WT	5'-GCAGCTTGAC*GGTACC*GCGCGTGAT-3'
2. E237A	5'-GAC*GGTACC*GCGCGTGATT**G**CCTGGGTGTCGTCG-3'
3. E237AR240A	5'-GAC*GGTACC*G**G**CCGTGATT**G**CCTGGGTGTCGTCG-3'
4. E237AD234A	5'-GAC*GGTACC*G**G**CCGTGATTTCCTGGGTG**G**CGTCG-3'

[a] Restriction sites are italicised and underlined and substituted nucleotides are shown in bold format

substitution mutation and *EcoR*I and *Sal*I sites on its ends. This strategy has been summarised in **Figure 2.2.**

2.4 Construction of KorB deletion mutants

N-terminal deletions were obtained by amplification by PCR using designed primers placing an ATG start codon in-frame with the remaining part of the protein. C-terminal deletions were also obtained by amplification by PCR using primers but by placing the stop codon in-frame with remaining part of the protein. Internal deletions were created by introducing *BamHI* site at 255 aa position of KorB and then 10, 20, 30 aa deletions were made upstream and downstream of the *BamHI* site to obtain KorB Δ225-255 aa, Δ235-255 aa, Δ245-255 aa, Δ255-285 aa.

2.5 Preparation of competent cells

An overnight culture of the *E. coli* strain was diluted to 1/100 in 25 ml of L-broth and incubated at 37°C (with shaking) until they reached mid-log phase (OD_{600} nm is 0.4 - 0.6); cells at this phase produce more competent cells. It usually took about 1.5 to 2 h to reach mid-log phase, then the cells were harvested by centrifugation at 4500 × g for 7 min at 4°C; the supernatant was removed. The pellets were resuspended in 10 ml of ice-cold 100 mM calcium chloride and put on ice for 20 min. The centrifugation step at 4500 × g, 4°C for 7 min was repeated and the pellets were carefully resuspended in 2.5 ml of ice-cold 100 mM calcium chloride. In order to freeze competent cells and store at -80 °C, the second resuspension step was carried out with 2.5 ml of ice-cold 100 mM calcium chloride with 15% glycerol; 100 µl samples were aliquotted into 1.5 ml eppendorf and frozen by dropping into liquid nitrogen.

2.6 Transformation of bacterial cells

The phenomenon by which bacteria take up foreign DNA is called transformation. Transformation can be performed only with competent bacteria. Depending on the concentration of the DNA, 0.5 – 10 µl of DNA was added to 100 µl of competent cells in a sterile 1.5 ml microfuge tube and mixed gently and then placed on ice for 30 min. The cells were then 'heat-shocked' in a 42°C water bath for 2 min. After incubation, 1 ml of L-broth (with no selective antibiotic) was added to each tube and the contents gently mixed. The tubes were then incubated for 1 hour at 37°C. 100 µl of the bacterial suspension was then plated out onto selective agar plates and incubated at 37°C overnight.

2.7 Isolation of plasmid DNA

2.7.1 Small scale isolation –alkaline lysis (miniprep)

The Alkaline lysis method, adapted from the technique described by (Birnboim and Doly, 1979), separates plasmid DNA from bacterial chromosomal DNA due to the unique property of plasmid DNA: it rapidly re-anneals after denaturation. This technique enables isolation of many plasmids in parallel for screening.

Overnight cultures were grown in 5 ml of L-broth (supplemented with appropriate antibiotics). 1.5 ml of the cell culture was transferred to an eppendorf tube and centrifuged for 1 min at 12,000×g in a microcentrifuge. If necessary a second 1.5 ml volume was added and harvesting repeated to double the quantity of bacteria being used. The supernatant was discarded and pellet was resuspended thoroughly in 100 µl (200 µl for double quantity) of lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) and tubes were placed on ice for 5 min. After incubation, 200 µl (400 µl for double quantity) of the freshly prepared 1:1 NaOH-SDS (0.4 M NaOH, 2% w/v SDS) solution was added, the tube was mixed by vortexing and placed on ice. After 5 min 150 µl of 3 M sodium acetate pH 5.0 (3 M sodium acetate, 11.5% v/v acetic acid, pH adjusted to 5.0 with glacial acetic acid) was added and the tubes were mixed by inverting. The mixture was spun at room temperature in a microcentrifuge for at least 5 min at maximum speed (12,000xg) and the supernatant was transferred to a new tube containing 400 µl of isopropanol without disturbing the precipitate. The tube was gently inverted to mix its contents prior to centrifugation (10 min at 12,000xg at room temperature). The supernatant was discarded carefully and the pellet was resuspended by vortexing in 100 µl of TNE (100mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA). Then 120 µl of isopropanol was added and the suspension was

mixed by inverting before centrifugation as in the previous step (10 min at 12,000x g at room temperature). The supernatant was carefully poured off, the tube was reverted and gently tapped on the paper towel to ensure that all excess liquid was removed, and then the pellet was dried at 48°C until all liquid was evaporated (15-30 min). The dried pellet was gently resuspended in 20 to 50 µl of $^{1}/_{10}$ TNE, TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA) or sterile distilled water.

2.7.2 Isolation of plasmid DNA "wizard prep"

The Wizard® *Plus* SV Miniprep DNA purification system kit (Promega Corp, USA) produces a high quality plasmid DNA sample that is used mostly for DNA sequencing. The centrifugation method was carried out according to manufacturer's instructions. All solutions were supplied by the manufacturer.

Approximately 5 ml overnight cultures were grown and 1-5 ml (1-5 ml for high copy number plasmid and 10 ml for low copy number plasmid) culture was pelleted by centrifugation for 2 min at 12,000×g in a microcentrifuge. The supernatant was removed and the pellet resuspended in 250 µl of the "Cell Resuspension Solution", by vortexing. 250 µl of the "Cell Lysis Solution" was added and the tubes inverted to mix the suspension. 10 µl of the "Alkaline Protease Solution" was added; the tubes were inverted 4 times and incubated at room temperature for 5 min. Next, 350 µl of the "Neutralisation Solution" was added and the tubes were inverted 4 times before centrifuging for 10 minutes at 12,000×g in a microcentrifuge. The cleared lysate was transferred to the spin filter (inserted in a collection tube) and centrifuged for 1 min at 12,000×g. The flow through was discarded and 750 µl of "Column Wash Solution" (previously diluted with 95% ethanol) was added to the column. The tubes were centrifuged for 1 min at 12,000×g and the

wash step repeated with 250 µl of "Column Wash Solution". The tubes were centrifuged for 2 min at 12,000×g (room temperature) to dry the pellet. The spin column was then transferred to a new tube and the DNA eluted by adding 100 µl of nuclease free water and centrifuging for 1 min at 12,000×g.

For low copy plasmids, 10 ml of culture was pelleted and the same method was followed, using 300 µl "Cell Resuspension Solution", 300 µl "Cell Lysis Solution", 12 µl "Alkaline Protease Solution" and 420 µl of "Neutralisation Solution".

2.7.3 Isolation of very low copy number plasmids –Midi-prep

The QIAGEN Plasmid Midi Kit was used to purify very low copy number plasmids, i.e. IncP α and IncQ and pSC101 replicon plasmids. This was carried out according to the manufacturer's instructions, using the solutions provided.

A 5 ml starter culture was inoculated with a single colony and then incubated at 37°C for 8 hours, with shaking. This culture was diluted by 1:500 in 400 ml of LB and incubated at 37°C for 12 hours, with shaking. The cells were harvested by centrifuging for 15 min at 6000×g, 4°C in a Beckman Avanti™ J-25 Ultracentrifuge (JA-10.500 rotor). The pellet was resuspended in 20 ml of QIAGEN buffer P1, with added RNase. 20 ml of QIAGEN buffer P2 was added; the solution was mixed and incubated at room temperature for 5 min. Next, 20 ml of ice cold QIAGEN buffer P3 was added and the solution mixed and incubated on ice for 30 min. The mixture was centrifuged for 30 min at 20,000×g, 4°C. The supernatant, containing the plasmid DNA was filtered over pre-wetted filter paper. 42 ml of room temperature isopropanol was added to precipitate the DNA and the mixture was centrifuged for 30 min at 15,000×g, 4°C. The pellet was

re-dissolved in 500 µl TE (10mM Tris-HCl, pH8 and 1mM EDTA, pH8) and 4.5 ml of QIAGEN buffer QBT was added.

The solution was added to a QIAGEN tip-1000, which had been previously equilibrated with 4 ml of QIAGEN buffer QBT. The column was washed twice with 10 ml of QIAGEN buffer QC and the DNA was eluted with 5 ml of buffer QF. 3.5ml of isopropanol was added to precipitate the DNA and the solution was centrifuged for 30 min at 50,000×g, 4°C. The pellet was washed with 2 ml of 70% ethanol and re-centrifuged at 15,000×g, 4°C for 10 min. The supernatant was carefully removed and the pellet air-dried. The DNA was resuspended in 0.5 ml of 10 mM Tris-HCl, pH8. After determination of the DNA concentration by agarose gel electrophoresis, the stock was stored at –20°C.

2.7.4 Large scale isolation of plasmids –Maxi-prep

The Maxi-prep method is based on large-scale alkaline lysis (described by Birnboim and Dolly, 1979), followed by caesium chloride/ethidium bromide density gradient centrifugation (Sambrook et al, 1989), which separates the plasmid DNA from the chromosomal DNA, after which the ethidium bromide is removed.

A 5 ml starter culture was set up in universals with appropriate selection and grown at 37°C for 5 hours, with shaking. 1 ml of this starter culture was used to inoculate 400 ml L-Broth (with the appropriate selection) and the bacteria were grown overnight at 37°C, with shaking. The overnight culture was centrifuged for 10 min at 6000×g, 4°C, to pellet the cells. The pellets were re-suspended in 20 ml lysis buffer. 50 ml of fresh 0.4 M sodium hydroxide-2% SDS in a 1:1 ratio

was added to this and the suspension mixed by gentle inversion and left on ice for no longer than 10 min. After incubation, 37.5 ml of 3 M sodium acetate pH 5 was added and the tubes inverted 8 times to mix. After incubating on ice for 15 min, the tubes were centrifuged at 10,000×g for 15 min at 4°C. The supernatant was strained through 1mm Whatman filter paper into 200 ml pots containing 100 ml isopropanol.

The tubes were inverted to mix the contents and then centrifuged at 10,000×g for 15 min at 4°C to pellet the DNA. The supernatant was removed and 4 ml of 70% ethanol was added to the tubes, which were then centrifuged at 10,000×g for 5 min at 4°C. After drying at 48°C, the pellets were re-suspended in 4 ml TNE and the tubes centrifuged for a further 10 min at 10,000×g at 4°C. Caesium chloride was dissolved into the supernatant (4.62g per 400 ml culture) and ethidium bromide (0.5 ml of 10 mg/ml per 400 ml culture) was added. The solution was transferred to a Beckman tube (3.5 ml Polyallomer Bell-top quick seal) using a Pasteur pipette (two tubes per 400 ml culture) and the volume increased to the top using TNE. The tubes were heat sealed and centrifuged at 100,000 rpm overnight on a Beckman TL-100 ultracentrifuge (424,480-343,828×g).

The bands were visualised using UV and the DNA was collected using a wide bore needle, minimising the exposure time of the DNA to the UV light. The plasmid DNA should be the lower band and the upper band is the chromosomal DNA. The samples were pooled and pipetted into a new Beckman tube and the volume increased to the top with the caesium chloride, ethidium bromide and TNE solution (4.62g CsCl, 0.5 ml ethidium bromide, made up to 7 ml with TNE). The tubes were centrifuged and the lower band collected as above.

The ethidium bromide was removed by the addition of an equal volume of water/caesium chloride saturated isopropanol. The tubes were mixed by flicking and the top, pink layer was removed. This was repeated until the top layer is clear. 400 µl or less of the plasmid DNA was transferred to an eppendorf (DNA was split into more tubes if the volume was over 400 µl). After the addition of 500 µl SDW to each tube, the tubes were centrifuged for 5 min at 14000×g. The supernatant was transferred to a new tube and the DNA precipitated by the addition of 530 µl isopropanol and 100 µl of 3M sodium acetate. The tubes were mixed and centrifuged for 5 min at 14000×g. After discarding the supernatant, the DNA pellet was re-dissolved in 200 µl TNE. 25 µl of 3M sodium acetate and 500ul ethanol was then added to precipitate the DNA once again and the tubes were centrifuged for 15 min at 14000×g. The supernatant was discarded and the pellet dried at 37C, before being re-suspended in 100 µl of 1/10 TNE.

2.8 Ethanol precipitation of DNA

Ethanol precipitation was used to concentrate DNA. $^{1}/_{10}{}^{th}$ of the volume of 3 M Sodium acetate pH5 and 2½ of the volume of absolute ethanol was added to the DNA. The solution was mixed by vortexing and incubated at room temperature for 10 min and then centrifuged for 15 min at 15,000×g, room temperature. The supernatant was carefully aspirated and the pellet was washed with 300 µl of 70% ethanol, before centrifuging for 10 min at 15,000×g, room temperature. The supernatant was carefully removed and the pellets dried at 48°C and resuspended in sterile distilled water.

2.9 Restriction digestion of DNA

Restriction digests were carried out using enzymes from: New England Biolabs®, MBI Fermentas and Invitrogen™. The digests were performed using solutions and instructions provided by the manufacturers. Multiple digests were carried out if there was a compatible buffer for all the restriction enzymes to be used in the multiple digest; otherwise digests were performed sequentially, with heat inactivation of the first enzyme if possible or ethanol precipitation in between.

Generally, reactions were carried out in 20 µl total volume with 4-8 µl of DNA, 5 units of each enzyme, 2 µl of the compatible buffer, 2 µl of 10× BSA (when absent from the buffer), for 1 to 2 h at temperature recommended by the supplier. 1 to 3µl of the 10 mg/ml RNase A was usually added per 20 µl of the sample for miniprep DNA. If larger amounts of DNA solution were used volumes and concentration of enzymes were increased appropriately. The digests were analysed on agarose gels. For overnight digests much less enzyme were used.

2.10 Polymerase chain reaction (PCR)

PCR (Saiki et al., 1985) enables amplification of DNA by using a pair of primers, designed to anneal to DNA flanking the target DNA to be amplified. The two primers prime the DNA polymerase to synthesize DNA across the region of interest. PCR involve three basic steps as follows:

- Denaturation of DNA at high temperatures of 90-96°C.
- Annealing of the primers at 50-60°C.
- Extension, where the DNA is synthesised by DNA polymerase at 72°C.

These steps are repeated several times, enabling exponential synthesis of the target DNA.

2.10.1 Designing of PCR Primers

Primers were designed by considering following criteria:

- It should be 17-25 bp long to ensure specific binding
- Its G+C content should be 50% at least
- Its melting temperature (Tm) should be around 52-65°C
- Incapable of dimerizing (annealing to each other)
- Incapable of forming secondary structures
- Lower G+C content at the 3' end to avoid mispriming
- One extra base before restriction site at 5` ends of the primer.

Tm (melting temperature) of primers was calculated by the following equation:

$$Tm\ (°C) = 59.9 + (0.41 \times \%GC)$$

Once primers were designed according to the criteria mentioned above, their quality was also determined by using the web site "Net Primer" (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).

2.10.2 Isolation of template DNA for PCR

Template DNA used in PCR reactions was either wizard prep, midiprep DNA or DNA prepared by a boil-prep method, as follows.

A fresh colony was picked from a L- agar plate, using a pipette tip and swirled into an eppendorf with 30 µl of distilled water. The mix was vortexed and boiled for 10 min and then spun in a microcentrifuge at 12,000×g for 5-10 min; the supernatant was removed immediately and 1 µl used in PCR reactions.

2.10.3 KOD HiFi DNA polymerase (Novagen)

Kod HiFi DNA Polymerase was used to get precise and accurate PCR products. The PCR reaction and procedure was carried out according to the manufacturer's instructions, using the solutions provided.

0.5 ml thick walled PCR tubes were set up for PCR reaction as follows:

5 µl 10× buffer #1 for KOD HiFi DNA polymerase

5 µl dNTP (2 mM)

2 µl $MgCl_2$ (25 mM)

4 µl each of the forward and reverse primer (5 pmol/µl)

1-4 µl template DNA

0.4 µl KOD DNA polymerase (2.5 u/µl)

Total volume was made up to 50 µl with distilled water.

Tubes were mixed and quickly spun down before transferring them to the PCR thermocycler. PCR reactions were carried out in the PCR Sprint Machine or Omn-E (Hybaid), according to the following program:

Denature	98°C for 15 sec	**25 cycles**
Anneal	(Tm –5)°C for 2 sec	
Extend	20 sec at 72°C	

2.10.4 BIO-X-ACT™ (Bioline)

BIO-X-ACT™ (Bioline) is a combination of a high fidelity DNA polymerase and Taq polymerase that produce comparatively accurate DNA fragments with A-tails for cloning into pGEM®-T easy.

0.5 ml thick walled PCR Tubes were set up for PCR reaction as follows:

5 µl 10× Opti buffer

1 µl dNTP (100 mM)

2.5 µl $MgCl_2$ (50 mM)

4 µl each of the forward and reverse primer (5 pmol/µl)

1-4 µl template DNA

1 µl BIO-X-ACT long polymerase (4 u/µl)

Total volume was made up to 50 µl with distilled water.

Tubes were mixed and quickly spun before being transferred to the PCR thermocycler. PCR reactions were carried out in the PCR Sprint Machine or Omn-E (Hybaid), according to the following program:

Denature	**94°C for 2 min**	**1 cycle**
Denature	**94°C for 1 sec**	**10 cycles**
Anneal	**(Tm –5)°C for 30 sec**	
Extension	**72°C for 1 min**	
Denature	**94°C for 15 sec**	**20 cycles**
Anneal	**(Tm –5)°C for 30 sec**	
Extend	**72°C for 1 min***	
Extend	**72°C for 7 min**	**1 cycle**

(*Increasing the time of the 72°C elongation stage by 5 sec each cycle)

2.10.5 Taq polymerase (Invitrogen™)

This was used mainly for A-tailing of the purified PCR products from PCR with KOD polymerase.

0.5 ml thick walled PCR Tubes were set up as follows:

10 µl 10× buffer

2 µl dNTP (100 mM)

2 µl $MgCl_2$ (100 mM)

50 µl High Pure PCR products

1 µl Taq polymerase

Total volume was made up to 100 µl with distilled water.

The tubes were mixed and spun down, and PCR reactions carried out at 72°C for 10 min.

2.11 Purification of PCR products using "High pure PCR product purification kit"

The High Pure PCR Product Purification Kit (Roche) was used to purify PCR products generated using Expand High Fidelity DNA polymerase (Roche) or Bio-X-Act DNA polymerase (Bioline). Purification was carried out as recommended by the manufacturer using reagents provided.

Usually a volume of PCR product is made up to 100 µl by adding the distilled water. Then 500 µl of "High Pure Binding buffer" was added to 100 µl of PCR product solution in the 0.5ml eppendorf and mixed well. The mixture was then transferred to a "High Pure filter tube" and spun at maximum speed for 30 to 60 sec. Flowthrough was discarded and 500 µl of "High Pure Wash buffer" was added to the filter. The tube was then spun at maximum speed for 1 min in a microcentrifuge (12,000 x g). The wash step was repeated using 200 µl and flowthrough was poured off again. Then 50 to 100 µl of "High Pure Elution buffer" was added to the filter and the tube was spun again for 1 min in a microcentrifuge (12,000 x g). Purified DNA was stored at -20°C.

2.12 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments (0.5-20kb), according to their relative sizes (Norton, 1986). Typically 0.6% - 2% w/v agarose gels (according to the size of DNA fragment to be separated) were made in 1× TAE buffer (50× stock solution, containing 2 M

tris-acetate 50 mM EDTA). Then 4µl ethidium bromide (10 mg/ml) was added to the molten agarose. Ethidium bromide is a fluorescent dye that intercalates between the DNA double helix. The mixture was then poured onto the tray and allowed to cool down . Then 6× DNA loading buffer (0.25% w/v bromophenol blue and 15% w/v ficoll) was added to the DNA sample before running it on the gel. RNae A was added in the miniprep DNA sample before loading on the gel, whereas in the case of wizard prep and midi prep it is already present in the manufacturer solutions. 10µl of 1 kb or 100bp ladder (NEB®) was also run to allow estimation of the size and concentration of the DNA run. Gels were run at 120V in 1× TAE buffer. The DNA was visualised using a BioRad Gel Doc 2000 transilluminator, which uses ultraviolet light to fluoresce the ethidium bromide. Software used was Quantity One Version 4.3.1 (BioRad) to visualize as as well estimate the quantity of DNA.

2.13 Purification of DNA from agarose gels

DNA fragments of 190 bp to 11 kb can be purified from agarose gels using the GENECLEAN® Spin Kit (BIO101, Carlsbad CP, and USA). It was used to purify less concentrated DNA from agarose gels, as its elution volume is small as well as in those cases where it was important to separate PCR product from the template DNA and any other amplified fragments because of annealing of primers on DNA around non targeted DNA . This method was mostly used to cut out the required DNA fragment from the restriction digested DNA sample from the agarose gel. The procedure was carried out according to the manufacturer's instructions, using the solutions provided.

The appropriate DNA bands were identified and cut from the gel. The GENECLEAN® SPIN GLASSMILK was resuspended and 400 µl was pipetted to GENECLEAN® SPIN Filter. The gel

slice was added to this (maximum 300 mg) and the gel was melted by heating to 55 °C in a water bath for 5 min; the tubes were inverted every minute during this incubation. When the gel melted, the liquid was spun out of the filter tube and into a Catch Tube by centrifuging in a microcentrifuge for 1 min at 12,000×g. The DNA was washed with 500 µl of GENECLEAN® SPIN NEW Wash (with added ethanol) and then the tubes were spun for 30 sec at 12,000×g. This wash step was repeated and the DNA pellet dried by centrifuging for 2 min. The filter was put into a new tube and the DNA was eluted by adding 15 µl of GENECLEAN® SPIN Elution Solution and re-suspending the GLASSMILK by carefully pipetting up and down, before centrifuging for 1 min at 12,000×g. This was repeated with 15 µl of Elution Solution.

2.14 Ligation of DNA

Two digested and separated fragments of DNA which were to be ligated together should have compatible ends generally created by appropriate restriction digest. Once insert and plasmid DNA fragments with compatible ends were obtained and their concentration was calculated using Quantity One software. Ligation reactions were performed using a 3:1 ratio of insert:vector for optimum insertion; the concentrations of both insert and vector were estimated using gel electrophoresis. The following formula was used to determine the volume of insert and vector required:

$$\text{ng insert} = \frac{\text{ng vector} \times \text{kb size insert}}{\text{kb size vector}} \times \text{molar ratio insert:vector}$$

The insert DNA was ligated to the digested plasmids using either the Quick Stick DNA Ligation Kit (Bioline USA, Inc) or T4 ligase (Invitrogen™). Ligase catalyses the formation of a

phosphodiester bond between adjacent 5'-phostphate and 3'-hydroxyl groups in either a blunt or 'sticky' ended configuration (Enrel, 1982).

2.14.1 T4 Ligase (Invitrogen™)

Reactions were set up in an eppendorf, as follows:

4 µl 5× buffer

1 µl T4 DNA Ligase (1 u/µl)

X µl vector DNA

X µl insert DNA

Total volume was made up to 20 µl by adding distilled water

Reactions were incubated for 24 hours at 14°C to ensure ligation. 5 µl of the ligation reactions was used to transform DH5α.

2.14.2 Quick Stick Ligation Kit (Bioline)

The Quickstick DNA ligase is a mutated form of T4 DNA ligase. The Quick Stick buffer enables fast ligation in 5 min at room temperature. Reactions were set up as above with manufacturer's solutions and incubated 5 minutes for sticky ends and 15 minutes for blunt ends, at room temperature.5 µl of the ligation reactions was used to transform DH5α.

2.14.3 pGEM-T easy (Promega)

The pGEM-T easy (Promega) is a T-tailed vector, used to clone and sequence A-tailed PCR products. It has MCS before T-tailing that can be used to cut out to have required sticky ends for the ligated A-tailed PCR product.

To ligate PCR products into pGEM-T, tubes were set up as follows:

5 µl 2× rapid ligation buffer

1 µl T4 DNA Ligase (3 u/µl)

1 µl pGEM-T easy vector

X µl insert DNA

Total voulume was made up to 10 µl by adding sterile distilled water

5 µl of the ligation reactions was used to transform DH5α. Transformation mixture was plated out on X-gal (L-agar) plates to get blue/white colonies (generally, white colonies are those that has got insert and blue ones without insert):

100 µg/ml	**ampicillin**
0.5 mM	**IPTG**
80 µg/ml	**X-gal (made up 50 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N,N-dimethyl-formamide)**

Insert DNA was sequenced from pGEM-T using universal primers (M13 forward; -40, and M13 reverse: -48) as pGEM-T has universal binding sites at either side of its *MCS*. pGEM-T is a high copy number plasmid and thus it is very easy to get more amount of high quality DNA to make sequencing more efficient. The universal primers used for sequencing purpose are as follows:

M13 forward (-40): 5'-GTTTTCCCAGTCACGAC-3'

M13 reverse (-48): 5'-GGATAACAATTTCACACAGGA-3'

2.15 DNA sequencing

Double stranded DNA sequencing was performed via the Sanger dideoxy-chain termination method (Sanger et al., 1977), using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Kit, Version 3 (Perkin-Elmer).

Reaction tubes were set up as follows:

200-500 ng	**template DNA**
1-3 µl	**appropriate primer (10 pmole/µl)**
8 µl	**terminator ready mix (diluted with buffer)**

Total volume was made up to a total volume of 20 µl by adding sterile distilled water.

Primer extension was carried out in a PCR thermocycler, PCR Sprint or Omn-E (Hybaid).

The program for sequencing is:

Rapid thermal ramp to 96°C

96°C for 30 sec

Rapid thermal ramp to 50°C

50°C for 15 sec

Rapid thermal ramp to 60°C

60°C for 4 min

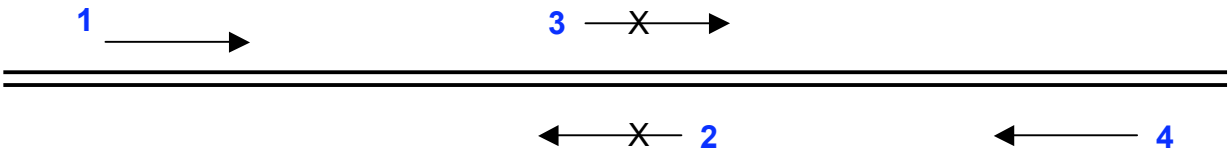
Rapid thermal ramp to 4°C

} **25 Cycles**

After PCR cycling, the sequencing reactions were purified to remove unincorporated dye terminators, which could vague data in the early part of the sequence and interfere with base scaling. The method used ethanol, to precipitate and pellet the DNA. The reaction mix was pipetted into an eppendorf and 2 µl EDTA (250 mM) and 64 µl of non-denatured 95% ethanol was added; the tubes were vortexed to mix the contents and left at room temperature for 15 min. After the incubation, the tubes were centrifuged at room temperature, for 20 min at 18,000×g in a microcentrifuge; the supernatant was removed (by carefully pipetting). Subsequently, 250 µl of 70% ethanol was added to wash the pellet and the tubes were centrifuged in the same orientation as previously for a further 10 min at 18,000×g, room temperature. The supernatant was removed as before and the tubes dried at 48°C. The DNA pellet was re-suspended in 10 µl deionised formamide (HiDi) before loading onto sequencing plate. Sequencing was carried out using a 3700 DNA analyser (Applied Biosystems).

2.16 *In vitro* overlap PCR

It is a method for rapid and easy mutagenesis internal to a DNA fragment (Ling and Robinson, 1997).



1st PCR

Two overlapping, mismatched, internal primers (**2** & **3**) were used in separate reactions with corresponding external primers (**1** & **4** respectively) on wild-type template. Products were purified from agarose (to get rid of the original template) using GeneClean (Bio101) HiPure (Boehringer-Mannheim) or similar kit and yield was checked on agarose gel. Primers **2** & **3** should overlap by at least 10 bp and should have the mismatched region at least 5-10 bp away from the 3' end (depending on number of mismatched nucleotides)**.**

2nd PCR

In the first 2 cycles of PCR, a small amount (e.g. 1 µl) of purified overlapping PCR products were used from **1st PCR** as template / primers. Then cycling was stopped, and external primers (**1** & **4**) were added as in **1st PCR** and thermal cycling program was restarted from the beginning.

N.B. All PCRs were performed with proofreading polymerase (e.g. Pfu, KOD) to minimise unwanted errors .

2.17 SDS-polyacrylamide gel electrophoresis

SDS-Polyacrylamide Gel Electrophoresis was first developed by (Davis, 1964; Ornstein, 1964) and is used to separate proteins according to their molecular weight. The protein sample is denatured by heating to 100°C in a SDA PAGE buffer that contains SDS and 2-mercaptoethanol that causes the proteins to unfold and become more rod-shaped. SDS binds to the protein, giving it a negative charge. Because SDS binding is proportional to the molecular weight of the protein, this gives the protein a uniform charge-to-mass ratio. This is how proteins are separated

according to molecular weight. The SDS-PAGE buffer system is a discontinuous buffer that contains SDS (sodium dodecyl sulphate).

SDS PAGE consists of two layers: a stacking gel (upper gel) and a resolving gel (lower gel). The stacking gel (pH 6.8) is more porous due to a low polyacrylamide concentration and thus proteins are concentrated into a stack, between the leading and trailing ions. When the proteins reach the resolving gel (pH 8.3) that has higher polyacrylamide concentration, they get 'un-stuck' and thus separate.

Generally, 10% resolving gels were prepared as follows:

3.35 ml	**acrylamide (30% w/v acrylamide, 0.8% w/v bis-acrylamide stock)**
2.5 ml	**lower Tris (1.5M Tris-HCl pH 8.8, 0.4% SDS)**
4 ml	**distilled water**
120 µl	**APS (ammonium persulphate)**
10 µl	**TEMED (N,N,N',N'-tetramethylethylenediamine)**

and poured quickly between the glass plates up to 1 ml below the top. The gel was overlaid with isopropanol and allowed to polymerise completely.

The upper stacking gel (3%) was prepared as follows:

0.5 ml	**acrylamide (30% w/v acrylamide, 0.8% w/v bis-acrylamide stock)**
1.25 ml	**upper Tris (0.5 M Tris-HCl pH 6.8, 0.4% SDS)**
3.24 ml	**distilled water**
60 µl	**APS (ammonium persulphate)**

6 µl TEMED (N,N,N',N'-tetramethylethylenediamine)

and poured on top of the resolving gel (once the isopropanol had been removed and washed). The comb was inserted between the spacers at the top of the plate. This was allowed to polymerise for 30- min.

10 µl samples (usually re-suspended in 1% SDS) were mixed to 10 µl of 4 × sample loading buffer, containing 0.4 ml of 1% bromophenol blue, 2.5 ml upper Tris, 2ml glycerol, 5 M urea, 5 ml distilled water and 0.2 ml 2-mercaptoethanol (added before use). The samples were incubated at 100°C for 5-10 min before loading onto the gel.

Gels were run in buffer (24 mM Tris base, 250 mM glycine, 0.1% w/v SDS in 1 Litre) at 100 V for 1-2 hours, until the bromophenol blue reached the bottom of the gel.

2.18 Coomassie blue staining

Protein bands were visualised by Coomassie Blue staining. Protein gels were soaked in Coomassie Blue stain for overnight (1 tablet of PhastGel® Blue R was dissolved in 80 ml sterile distilled water, 120 ml methanol and 200 ml of 20% v/v glacial acetic acid). The gel was then destained for 1-2 hours in destain solution (10% v/v glacial acetic acid, 40% v/v methanol, 50% v/v distilled water). The destain solution was changed once during the process if required. Protein gels were then fixed into water containing glycerol for 30 min and then dried between cellophane sheets overnight.

2.19 Purification of His-tagged proteins

The His_6 tag is also named as an affinity tag as it provides affinity to the protein to bind the Ni-NTA. It has been reported that His_6 tag does not interfere with the structure and function of the protein but it is opposite in the case of WT KorB and its derivatives (Luckazewick et al., unpublished).

WT KorB and its derivatives were expressed from high expression vector pET 28a that expressed His tagged and T7 tagged protein from a T7 promoter. KorB and its derivatives were overexpressed in *E coli* BL21 ; a lysogen, in which the bacteriophage T7 gene is present on the chromosome. T7 gene expression is controlled by IPTG. Another host strain used for protein overexpression and its comparison with BL21 was BL21 pLysS which is the same as BL21 but contains the pLysS plasmid that can provide additional stability for the target genes expression in the host strain, only if it is compatible with the incoming expression vector. The pLysS plasmid is used to suppress basal transcription of T7 polymerase before the induction of IPTG.

2.19.1 Over-expression

Overnight cultures were diluted by 1:50 in 400 ml LB (with kanamycin to select for pET28-a and chloramphenicol to select for pLys) and grown to mid-log phase (OD_{600nm} ~0.4-0.6). Once cells reached log phase, protein expression was induced by the addition of 0.1-1 mM IPTG and the bacterial suspension was incubated for a further 4-6 hours.. Cells were harvested by centrifuging for 20 min at 18,600×g, 4°C in a Beckman Avanti™ J-25 Ultracentrifuge (JA-10.500 rotor). The supernatant was poured off and the cells washed in 10 ml in STE (100 mM NaCl, 10 mM, Tris-HCl pH 8 and 0.1mM EDTA) and re-centrifuged. The supernatant was discarded and the pellet dried.

2.19.2 Determining the solubility of the protein

To examine whether the over-expressed protein was soluble or insoluble, 1 ml of the cell suspension was pelletted and resuspended in buffer (0.1M Tris-HCl pH 7.6) and sonicated (6× 10 sec bursts, amplitude10 microns). The samples were centrifuged in a microcentrifuge at 18,000 ×g for 20-30 min at 4°C. The supernatant was poured off and the pellet was resuspended in 1% SDS. Samples from both fractions were analysed by SDS-PAGE.

2.19.3 Purification using a nickel-agarose column

Purification was carried out using a Nickel-Agarose column (QIAGEN). Solutions were made and the method used according to the manufacturer's instructions. The pellet was re-suspended in 2-5 ml of lysis buffer per gram of cells (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidiazole, adjusted to pH 8 with NaOH). Lysozyme was added to a volume of 1 mg/ml and the mixture was incubated on ice for 30 min. After this time, mini EDTA-free protease inhibitor cocktail was added (1 tablet dissolved in 1.5 ml distilled water –Roche Diagnostics). The cells were sonicated on ice, using 6× 10 sec bursts at 10 microns amplitude, with a 10 sec cooling period. The cells were then centrifuged at 10,000×g for 20-30 min at 4°C in a Beckman Avanti™ J-25 Ultracentrifuge (JA-20 rotor).

1 ml of the 50% Ni-NTA slurry was added for every 4 ml of cleared lysate in a 10 ml tube; the tube was mixed gently on a rotary shaker at 200 rpm, 4°C for 60 min. The lysate Ni-NTA mixture was loaded onto a column. Once settled, the cap was removed and the flow through collected. The column was then washed twice with 4 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, adjusted to pH 8 with NaOH). The His-tagged protein was

eluted with 4 × 1 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, adjusted to pH 8 with NaOH).

10 µl samples from each stage were run on SDS-PAGE to ensure that the protein is not lost and is pure.

2.19.4 Thrombin cleavage of His-tagged proteins

To compare the function of His and non-His tag protein and to determine the effect of His tag, His tag was removed from the purified His tag proteins using thrombin cleavage. Thrombin is an endoprotease, which has a specific cleavage recognition sequence: LeuValProArg▼GlySer. This site is encoded between the his-tag and the N-terminus of the protein. Proteins were expressed from a modified pET28a vector in which the DNA encoding the T7 tag has been removed. Consequently, following thrombin cleavage, the following amino acid residues are all that remain at the N-terminus of the purified protein: GlySerHisSerGluPhe. Thrombin cleavage was carried out using Thrombin Kits from Novagen. The thrombin in these kits is biotinylated to enable removal from the protein, using Streptavidin agarose, following the cleavage.

To optimise conditions for cleavage, small-scale experiments were first carried out. Thrombin was serially diluted (1:25, 1:50, 1:100, 1:200) in Thrombin Dilution/Storage Buffer. Five tubes were set up, each with 10 µg of the target protein, 1 µl of each dilution of thrombin (one negative control tube with no thrombin), 5µl of 10× Thrombin Cleavage/Capture Buffer, made up to 50 µl with deionised water. The reactions were incubated at room temperature; 10 µl aliquots were taken after 2, 4, 8 and overnight incubations and analysed by SDS-PAGE.

Once optimal conditions were determined, the total reaction volume required was then established, since when scaled up the reaction volume became quite large. Tubes were set up with 1 µl of the optimal Thrombin dilution, 10 µg of the target protein and appropriate volumes of the 10× Thrombin Cleavage/Capture Buffer, and the total volumes adjusted to 10, 20, 30, 40 and 50 µl. Reactions were then scaled-up proportionately for large-scale thrombin cleavage.

Following cleavage, the biotinylated thrombin was removed by incubation with Streptavidin agarose (using a ratio of 32 µl of the slurry for each unit of enzyme). Tubes were incubated at room temperature for 30 min on a rotor. After incubation, the Streptavidin-agarose complexed with biotinylated-Thrombin was separated from the protein by spin-filtration: the reaction was transferred to the top of the spin filter and the tubes centrifuged for 5 min at 500 ×g. The filtrate, which consists of the cleaved protein free of the Thrombin, was kept.

2.19.5 Protein dialysis

Protein dialysis is used to alter the composition of buffer in which the protein is present. For this purpose semi-permeable membranes are used that have pores that are large enough to allow the free transport of small molecules (i.e. salts) but are small enough for the transport of large molecules.

The dialysis tubing was prepared according to the manufacturers instructions. The tubing was cut to appropriate size according to the volume of buffer in which protein is present, and soaked in distilled water for 30 minutes. Prior to use, it was rinsed thoroughly in distilled water. One end of the tubing was tightened with a plastic clamp. The protein was loaded into the tubing with a pipette and the top end of the tubing secured as above. The dialysis sample was placed into a

beaker with the appropriate dialysis buffer, which was approximately 100× the sample volume. Dialysis was continued overnight at 4ºC, with stirring.

2.19.6 Amicon ® ultra centrifugal filter devices (Millipore)

These devices are tubes with filters that prevent high molecular weight compounds (proteins) from passing through. They enable concentration of proteins by centrifuging the sample through the filter. After washing with SDW, upto 15 ml of sample was added to the filter unit. The tubes were centrifuged at 4000 ×g and 4 °C for 15-45 min, until the protein had been sufficiently concentrated.

2.19.7 Determining the protein concentration

Protein concentration was determined using the spectrophotometer. The OD_{260} and OD_{280} were measured in a quartz cuvette for the appropriately diluted protein in buffer.

Protein concentration was calculated by:

$$\text{Protein concentration (mg/ml)} = 1.55\ (A_{280}) - 0.76\ (A_{260})$$

2.20 Gel retardation assays

Specifically designed primers were used to amplify the target region

2.20.1 ^{32}P labelling of DNA fragments

Specifically designed primers for amplifying promoter regions were used to do PCR of the target region. PCR product runned on gel to separate it from the template and high purification of PCR

product was performed using High Purification Kit. Few microlitres of high pure PCR product run on gel and thus its concentration was determined using quantityone software. These DNA fragments were then labeled using T4 polynucleotide kinase (MBI Fermentas). The labelling reaction was prepared using the given volume of highly pure PCR generated DNA fragments, 2µl of PNK buffer A (for forward reaction), 2 µl of polynucleotide kinase, 1 µl of $\gamma^{32}P$ (7000Ci/mol)-final volume up to 20µl for 30 min at 37°C.

2.20.2 Binding reaction

^{32}P-labeled fragments of DNA were incubated with different concentrations of WT or mutant KorB protein extracts or purified KorB protein in a 20 µl final volume of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 10 mM DTT, 100 µg/ml of salmon sperm DNA and 100 µg/ml of BSA. Samples were spun to bring all ingredients to the bottom of the tube and incubated for 15 min at 37°C.

2.20.3 Band-shift assays

Electrophoresis was performed on 5% native polyacrylamide gels in TBE buffer (90 mM Tris-borate, 1 mM EDTA). The gels were run at 10V/cm for 3 h at 4°C, fixed for 10 min in 12% methanol/10% acetic acid and subsequently dried for 1 h at 80°C under vacuum. Dried gels were exposed overnight to K-screen (BioRad) and analyzed using a Phosphorimager system and QuantityOne system.

2.21 *xylE* assay

E. coli C600K bacterial strains containing a pPT01-derivative carrying a copy of the *xylE*, encoding catechol 2,3-oxygenase, under control of KorB regulated promoter as well as a plasmid providing KorB and/ KorA or TrbA *in trans* under control of the *tac* promoter were used in a two- or three-vector system. The overnight cultures were inoculated in 5 ml LB with appropriate antibiotics with a single colony taken from a fresh plate. Cultures were incubated at 37°C with shaking. On the following morning duplicate flasks containing 20 to 25 ml of selective LB were inoculated with the overnight culture at 1:100 dilution. Transcription from *tacp* was induced with 0.5 mM IPTG (two vector system) and incubated with shaking at 37°C until A_{650} = 0.4 - 0.6 which usually took 3 to 3.5 h. At mid-log phase cultures were collected, the bacteria harvested by centrifugation at 8,000 x g for 5min at 4°C. From this point onwards samples were always stored on ice. The supernatant was poured off and the pellet was resuspended by vortexing in 500 µl of a 9:1 mixture of 0.1 M sodium phosphate buffer pH 7.4 and acetone. The cell suspension was then transferred to a 1.5 ml microfuge tube and sonicated for 10 to 15 sec (amplitude 9 microns). The cell debris was spun down by centrifugation of lysates at 4°C for 15 min in a microcentrifuge at maximum speed (14,000xg). The catechol 2,3-oxygenase activity was determined spectrophotomerically by following the increase in absorbance at 374 nm due to the accumulation of 2-hydroxymuconic semialdehyde. The reaction cuvettes contained 2.8 ml of 0.1 M sodium phosphate buffer pH 7.4 and 200 µl of freshly prepared 3 mM catechol solution. 10 to 100 µl of the cleared lysate was added to the cuvette prior to measuring absorbance. One unit of catechol 2,3-oxygenase is defined as the amount needed to convert 1 µmol of substrate to product in 1 min under standard conditions. The activity of the catechol 2,3-oxygenase was calculated using the following equation:

$$\text{Activity} = \frac{\textit{xylE} \text{ activity} \times \frac{1000}{v}}{\text{Protein concentration}}$$

Where: v = volume of the cleared lysate added to reaction

xylE activity = increase in absorbance at 374 nm

Protein concentration was determined by the biuret method (Gornall et al., 1949) as described below.

2.22 Biuret assay

Protein concentration was determined using the biuret method. 1 ml reaction cuvette contained 200 µl of cleared lysate and 800 µl of biuret reagent (1.5g $CuSO_4 \cdot 5H_2O$, 6.0g $NaKC_4H_4O_6 \cdot 4H_2O$, 300 ml 10% NaOH and water up to 1 liter). Samples were incubated for 20 to 30 min in the dark at room temperature and then absorbance was measured at 540 nm.

2.23 Protein co-purification (pulldown assay)

BL21 cells carrying either *tacp*/T7 promoter expression plasmids were grown overnight in LB + antibiotic + 1% glucose. Next day cultures were diluted 1/100 and were grown to OD= 0.6-1.0 before induction with IPTG at 0.5 mM. After induction of IPTG, cultures were grown for 4 hours at 37 ºC with shaker at 200 rpm.

BL21 cells carrying *tacp* or T7 promoter constructs were mixed together in equal volumes (i.e. 1 ml each) and harvested. Cells were resuspended in 200 µl lysis buffer and incubated with lysozyme at 1mg/ml for 30 minutes. Cells were lysed by sonication for 15 seconds. Lysates were centrifuged for 10 minutes at 15,000×*g* to remove the cellular debris and supernatant was transferred to fresh tube. Ni-NTA 50 µl was added and mixed gently for 30 minutes at 4 ºC. Reactions were centrifuged for 10 sec at 500×*g* to pellet the agarose and supernatant was transferred to a fresh tube. The agarose was washed twice with 100 µl wash buffer and proteins were eluted with 60 µl elution buffer. Elutions and unbound protein from prepurification samples were checked on 15% SDS PAGE.

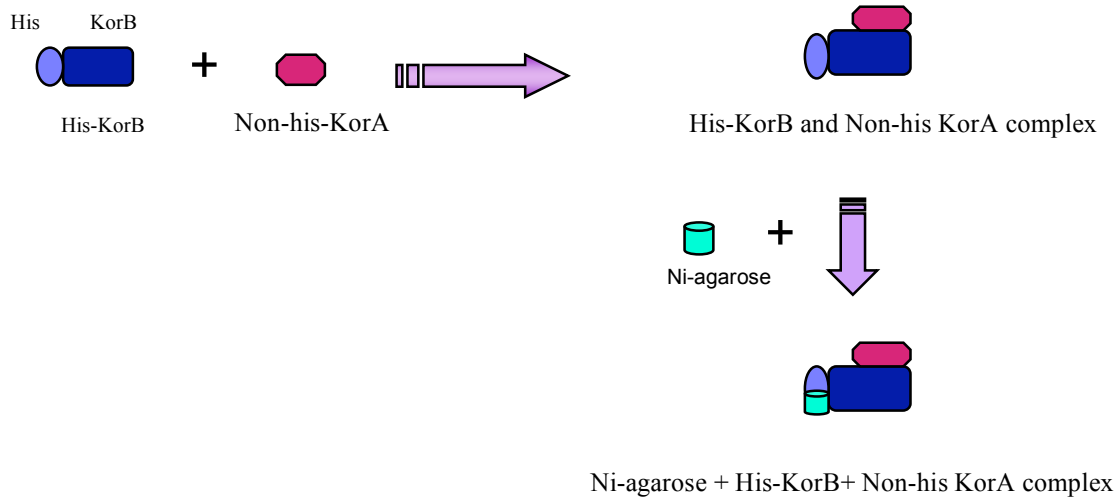


Figure 2.3: Purification of KorB-KorA complex in pulldown assays

2.24 Western blotting

Proteins were first separated by SDS polyacrylamide gel electrophoresis and then blotted onto a nitrocellulose membrane (Hybond-C pure) (Sambrook et al., 1989) using Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad). The transfer was carried out for 1 hour at 100V at 4ºC in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol). A prestained broad range protein marker (NEB®) was run along side samples, so that marker proteins could be seen on the membrane.

The blot was carried out using the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay (BioRad). The membrane was immersed at 45° into the blocking solution - 5% non-fat dry milk in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH .5). The solution was gently mixed at the room temperature for 1 to 2 h. Then the membrane was incubated with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween, pH 7.5) for 5 to 10 min with gentle agitation. The wash step was repeated twice. After washing the membrane was incubated for 1 to 2 h with gentle agitation with anti-KorB antibody diluted 1:1000 in TTBS. The washing step was repeated and the washed membrane was then incubated with biotinylated goat anti-rabbit antibody diluted in TTBS at the room temperature for 1 to 2 h with gentle agitation and then washed again with TTBS. After washing, the membrane was incubated with streptavidin-biotinylated alkaline phosphatase complex for 1 to 2 h at room temperature. The membrane was washed twice again before the incubation with colour developer. The blots were dried on Whatman paper overnight and then scanned and analysed using QuantityOne software.

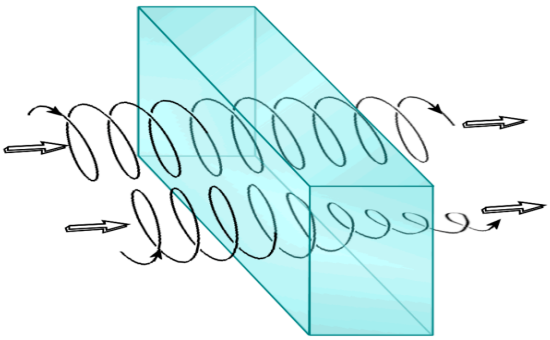
2.25 Circular dichroism

Circular Dichroism (CD) is the difference in absorption of left and right circularly polarised light. It is the ideal technique for studying chiral molecules in solution. The most common applications include proving that a chiral molecule has been synthesised or resolved into pure enantiomers and probing the structure of biological macromolecules, in particular determining the secondary structure (including a-helix and b-sheet) content of proteins.

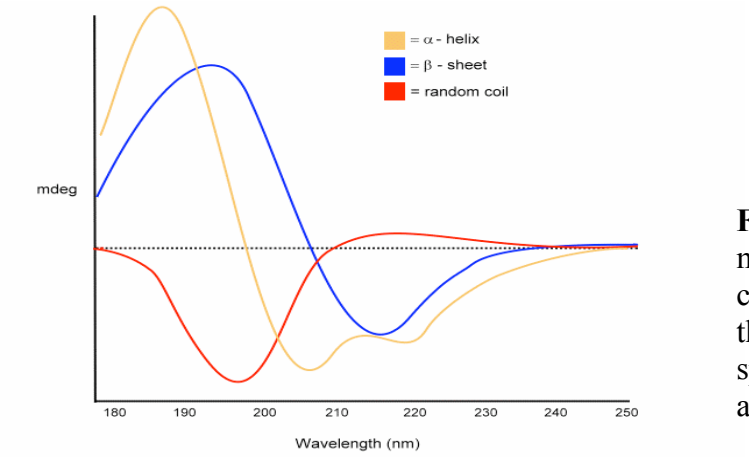
The CD spectrum of a protein molecule can be used to obtain structural information, for example about the arrangement of peptide bonds in secondary structure elements like helices and sheets. The amount of the different secondary structure elements can be obtained by fitting a CD spectrum to a set of known reference spectra.

Portions of linear chain of proteins fold into regular conformations i.e. a helix, b- sheet, b or reverse turns, omega loops, random coil. The α-Helix is a cylindrical shape. It consists of 10 -15 amino acid residues and 3-4 turns while each turn contains 3.6 amino acid residues covers distance of 5.41 Å. It consists of amino acids with extended side chains, and hydrophilic and hydrophobic amino acids lie on opposite faces of cylinder. The β-Sheet is a sheet-like structure. It consistis of 2-6 strands (3-10 amino acids each) stabilized by H-bonds (parallel and antiparallel which is more common). It contains amino acids branched at β-C e.g., isoleucine, threonine, valine. One example is silk fibroin (poly Ala-Gly) which has high β-sheet content (400,000 Da).

(a) CD machine



(b) Left handed and right handed circularly



(c) CD spectrum

Figure 2.4: Showing (a) CD machine, (b) left and right handed circularly polarised light used in the CD machine and (c) CD spectrum showing α-helix, β-sheet and random coiled structure.

2.26 Analytical ultracentrifugation

It measures the rate at which molecules sediment during centrifugation with high gravitational forces. Sedimentation depends on the mass and shape of the molecule and can be used to give information on molecular mass, conformation and oligomeric properties of proteins.

Sedimentation velocity experiments were carried out in a Beckman XL-A analytical ultracentrifugation (Beckman Coulter, Palo Alto, CA, USA) equipped with absorbance optics. Protein samples were dialysed overnight into buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.0) and then any precipitated protein cleared by centrifugation at 14,000×g and 4 °C for 30 min. Samples (400 μM, with protein concentration of approximately 0.1 mg/ml) were loaded into one channel of cells with two channel Epon centre pieces and sapphire window. The reference (420 μM of buffer alone) was loaded into another channel. Samples were centrifuged at 40,000 r.p.m., 4°C using an An50Ti rotor. Scans of an absorbance wavelength of 280 nm, were taken every 6 min. Partial specific volumes were calculated using the programme SEDNTERP (Laue et al*.,* 1992). The data was analysed using the programme SEDFIT (Schuck et al., 2000). Sedimentation coefficient distributions were calculated using the Lamn equation modelling, implementing maximum entropy regularization. 100 scans were analyzed for each sample, which represents the full extent of sedimentation of the sample.

Chapter 3: Role of the N-terminal domain of KorB in DNA binding

3.1 Introduction

KorB protein belongs to the ParB family of partitioning proteins and possesses an HTH binding motif (Motallebi-Veshareh et al., 1990). It has a predicted negative charge of –21, uncharacteristic of DNA-binding proteins, and a pI value of 4.27 (Pansegrau et al., 1994) yet it specifically recognizes a well defined DNA operator, O_B. The O_B sequence, 5'-TTTAGC$^{G}/_{C}$GCTAAA-3', occurs twelve times on the RK2 genome (O_B 1-O_B 12). These operator sites are located in three positions relative to RK2 promoters. Class I sites lie immediately upstream of the –35 region of the promoters (O_B1, O_B 10, O_B 12) at promoters *korAp, trfAp*, *klaAp* (Kostelidou and Thomas, 2000). Class II sites are found up to 189 bp upstream or downstream of a transcription start point (O_B 2, O_B 9, O_B 10, O_B 11) at promoters *kfAp*, *trbBp*, *trbAp* and *kleAp* (Kostelidou and Thomas, 2000). Note that O_B 10 is Class I relative to *trfAp* but Class II relative to *trbAp*.

The three dimensional structures of both the C-terminal and the central DNA-binding regions of KorB were recently solved separately (Delbrück et al., 2002; Khare et al., 2004). The CTD structure shows close similarity to an SH3 domain, which is well known in eukaryotic proteins involved in signal transduction (Delbrück et al., 2002). It forms a very stable dimer, the subunits interacting through a beta-ribbon with hydrophobic residues that create a pseudo-leucine zipper. Deletion mutations that affect this region result in a monomeric protein suggesting that this region is the primary dimerisation determinant and similar results have been observed in other ParB proteins. The region responsible for DNA binding lies in the middle of KorB – large

deletions from either N- or C-termini leave this activity intact, but C-terminal deletions in particular reduce DNA binding affinity (Lukaszewicz et al., 2002). The crystal structure of the DNA binding domain with DNA showed that despite loss of the CTD it can still bind DNA as a dimer (Khare et al., 2004), although it appears to be a monomer in solution. DNA docking depends on the region predicted to contain an HTH motif, but specific recognition is predicted to depend on protein-DNA contacts outside of this region. It was proposed that the interface between the DNA binding domains normally stabilises the protein-DNA complex. A similar conclusion was reached from the crystal structure of the N-terminal and central domains of Spo0J from *Thermus thermophilus*, another ParB homologue, that was recently determined (Leonard et al., 2004). It was suggested that the NTD may also be involved in dimerisation and a model was proposed in which this interface can either control the DBD interaction in a dimer, or may bring together two dimers at an operator, so that they may in turn facilitate binding of further dimers on either side and thus promote spreading outwards from the operator.

These structural studies start to make sense out of observations obtained in this laboratory over a number of years and that are relevant both to how KorB functions in partitioning and represses transcription from a distal binding site. The study started with a series of deletions in the N-terminus of KorB and the resulted proteins were tested for their interaction with DNA. The results in this Chapter demonstrate that the N-terminal region is an important modulator of the architecture of KorB-DNA complexes.

3.2 Results

3.2.1 Protein purification

KorB proteins were purified as described in Chapter 2. Protein purification conditions were optimised by using a range of IPTG concentrations for the induction of *t7p* to produce proteins. The best conditions for KorB (WT/mutant) were 1 mM IPTG at OD 4 of the culture grown from the diluted overnight culture. Reasonable amounts of clean proteins were obtained. KorB (WT/mutant) were very soluble and stable. Purified proteins were used DNA binding assays.

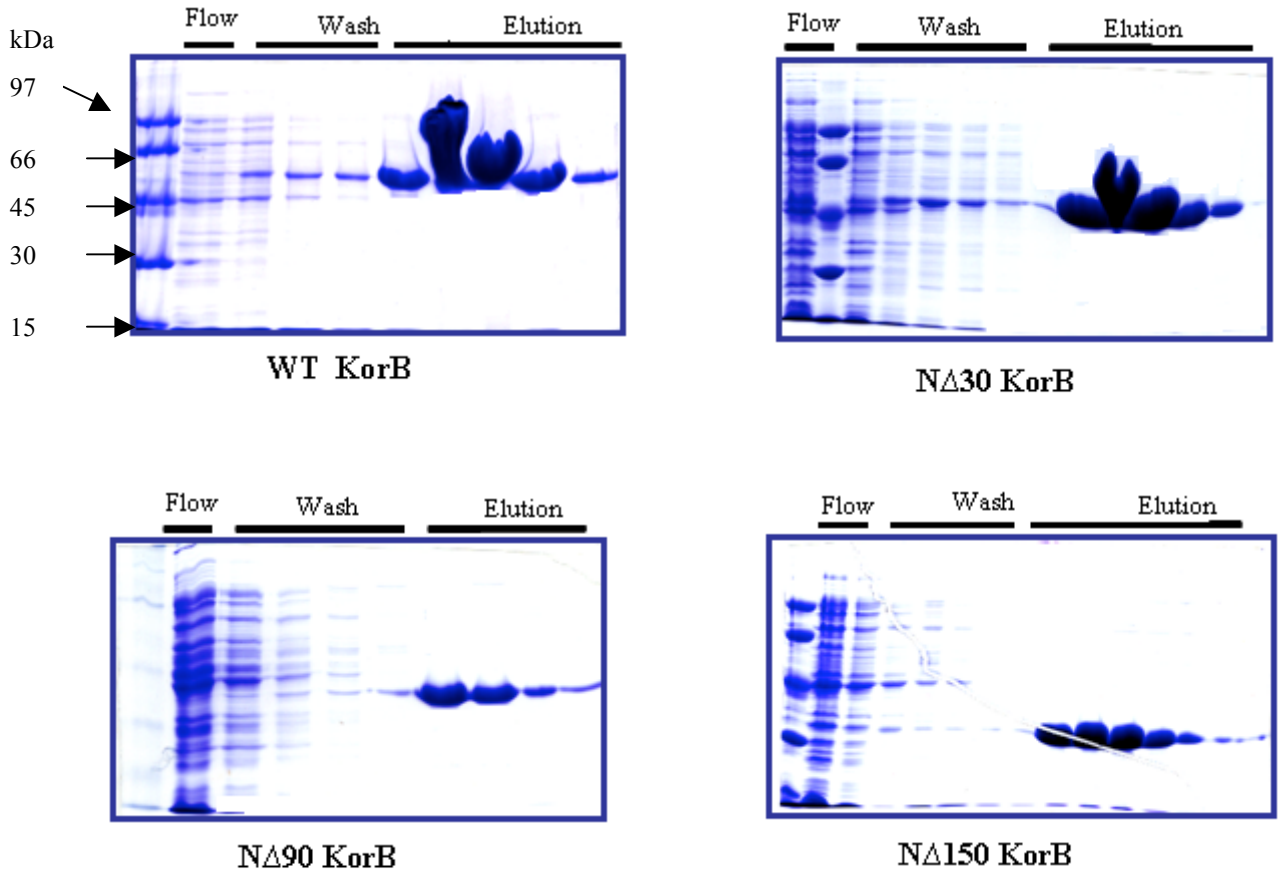


Figure 3.1: Purification of His-tagged KorB (WT/ N-terminal deletion mutants)

3.2.2 Circular Dichroism of KorB (WT/ deletion mutants)

The secondary structure content of His-tagged KorB (WT/ deletion derivatives) was compared by using circular dichroism (CD) spectra at 180 - 300 nm and 25˚C. The path length of the cuvette used was 0.5mm. About 100 µl samples of 0.5 mg protein solution (20 mM Tris, 100 mM NaCl, 10 mM EDTA, pH = 7) was loaded between thin cuvettes and the spectrum was obtained at 180-300 nm and 25˚C. Data obtained were plotted to make graphs using Microsoft Excell. Comparison of the spectrum with standard spectra **(Figure 3.2a)** measured for proteins with essentially 100% α-helical, β-sheet or random coil structure indicate that KorB is likely to be largely α-helical which is consistent with both predicted secondary structure and published crystal structure of KorB DNA binding domain and C-terminal domain. Spectra determined for the N-terminal mutant proteins purified during this study indicate that they each retain a spectrum similar to WT KorB as shown in **Figure 3.2b**.

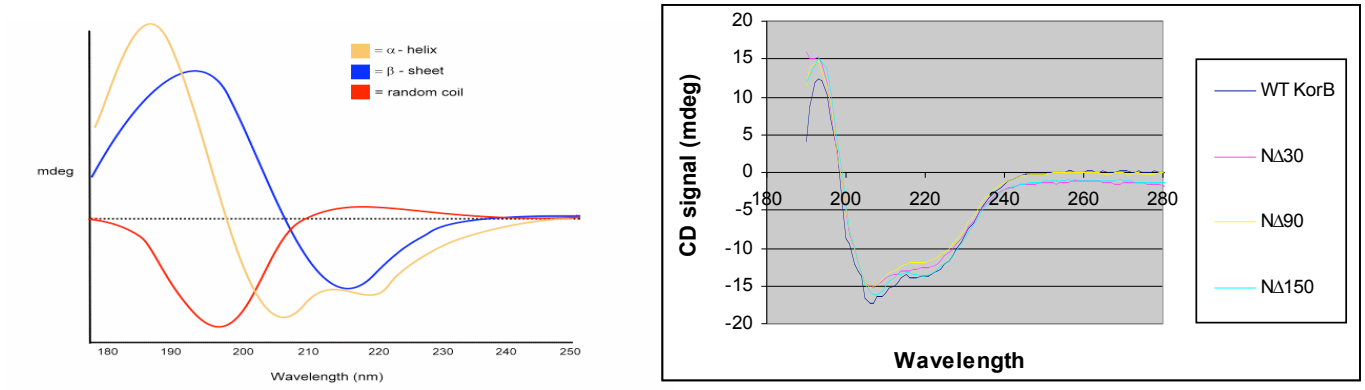


Figure 3.2 Circular Dichroism. (a) CD spectrum showing α-helix, β-sheet and random coiled structure. (b) CD spectrum of KorB (WT/ deletion derivatives) at 25°C.

KorB thermal denaturation was determined by using the temperature range from 20 to 90 °C at 220nm in circular dichroism (CD) machine. Proteins (0.5 mg) were loaded in the 0.5 mm cuvette and placed in the CD machine and data were obtained by computer linked to the CD machine. **Figure 3.3** shows that deletion of from 30 to 150 aa from the N-terminus of KorB did not change its thermal denaturation as WT and mutant KorB proteins were denatured over the same temperature range.

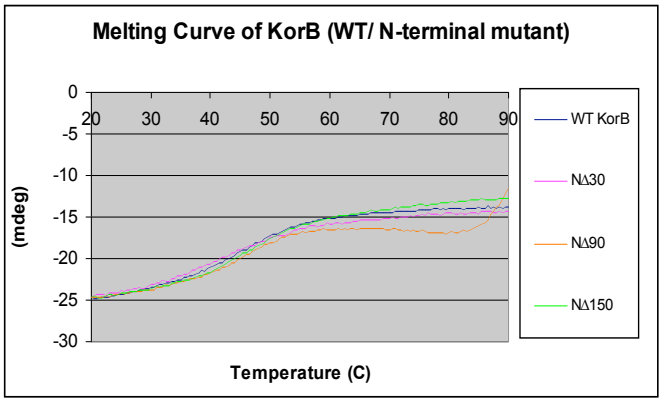


Figure 3.3: Thermal denaturation of KorB (WT/ deletion derivatives) using CD machine. Temperature was varied between 20 - 90 ˚C.

3.2.3 Role of His-tag on the binding affinity of KorB (WT or mutant)

N-terminally linked His_6 tagged KorB (WT/N-terminal deletion mutants) was used to determine the effect of the His-tag on KorB ability to bind DNA. Non-His tagged KorB proteins were obtained by removing the His-tag using thrombin cleavage. Proteins were dialysed overnight in buffer (50 mM Na_2HPO_4 and 300 mM NaCl, pH=8).

PCR-amplified fragments were used for electrophoretic mobility shift assays (EMSAs). These fragments were obtained using primers FT11 (5`-GCTTCCGGCTCGTATGTTG-3`) and FT22 (5`-CGAAAGGGGGATGTGCTGC-3`) on pKK113 template (pUC18 having O_B cloned between *EcoR*I and *Hind* III). PCR reactions were prepared and performed as described in **Chapter 2**.

Protein-DNA binding studies were performed using EMSAs. As a first step protein concentrations from 50 to 1000 nM were used to find the concentration range where KorB WT or mutants are shifting the DNA. As a second step, more reactions were made within the concentration range where protein was shifting 50% of the DNA to enable us to measure the binding constant (*Kapp*). The results obtained have been shown in **Figure 3.4.**

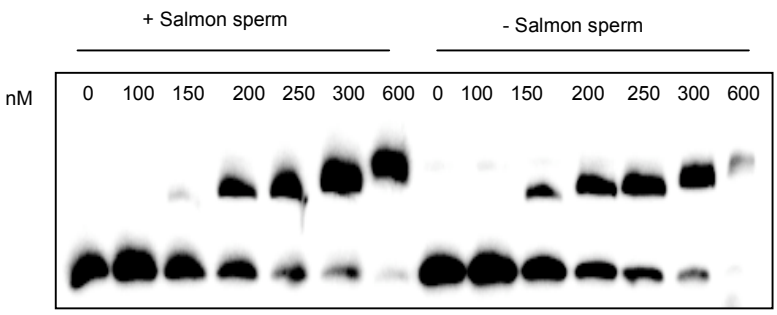
Figure 3.4 shows that all of the N-terminal KorB mutants bind DNA having O_B. Retardation gels were quantified using the QuantityOne software (Biorad). The concentration of retarded DNA fragment was plotted against protein concentration to estimate the apparent affinity to O_B site (*K*app- equal to a protein concentration giving 50% occupancy of the DNA). Binding of KorB (WT or N-terminal derivatives) was measured at varying protein concentrations, above and below *K*app. *K*app value of His tagged protein was calculated in the presence or absence of salmon sperm DNA to check the binding specificity of KorB (WT/mutant) with DNA having O_B.

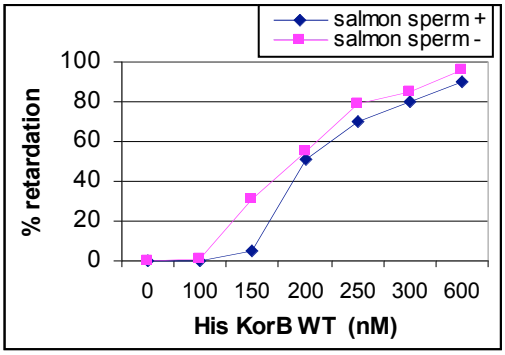
The calculated *Kapp* values are presented into **Table 3.1**. A comparison has been made between WT KorB and its deletion derivatives binding affinities in the presence and absence of His-tag and salmon sperm DNA. The *Kapp* values show that in the presence of competitive salmon sperm DNA His-tagged KorB (WT or deletion derivatives) has slightly lower affinity for the 180 bp DNA fragment having O_B. All of the KorB N-terminal deletion mutants (NΔ30, NΔ90 and NΔ150) have higher binding affinity for DNA compare to the WT, irrespective of the presence of salmon sperm DNA. Salmon sperm presence didn't make any difference on their DNA binding affinities of WT KorB **(Figure 3.4a & b)**.

Table 3.1: DNA binding constant (*Kapp*) of KorB (WT or N-terminal mutants) to 180 bp fragment having WT O_B

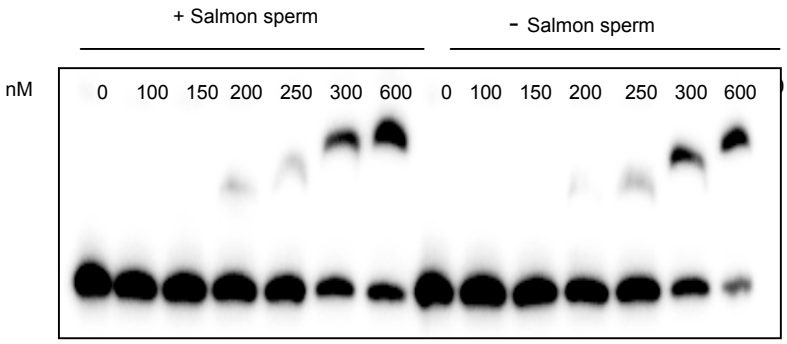
KorB (WT/ N-terminal mutants)	His-tagged or non His-tagged	*Kapp* in nM for O_B in the presence and absence of competitive	
		Salmon sperm (100µg/ml)	No salmon sperm
WT	+ –	200 300	200 300
NΔ30	+ –	100 130	80 130
NΔ90	+ –	90 110	80 80
NΔ150	+ –	80 80	50 80

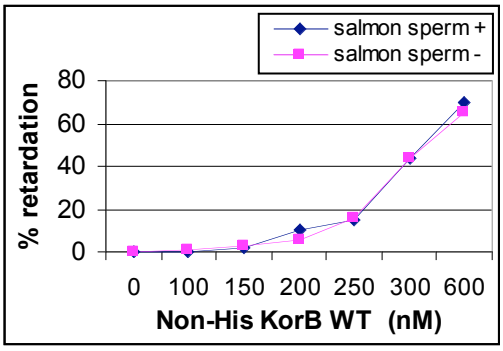
(a) His-tagged WT KorB



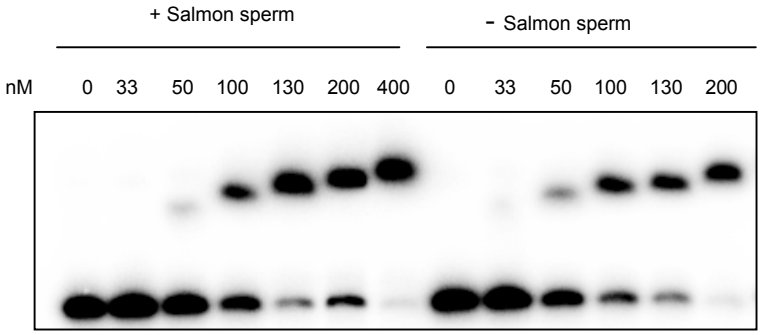


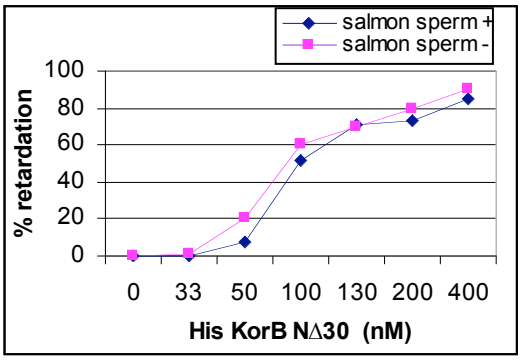
(b) Non His-tagged WT KorB



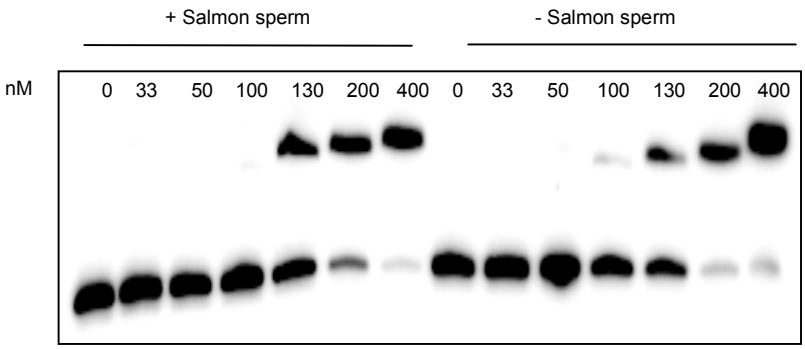


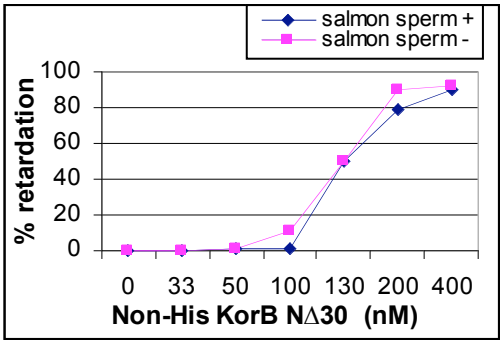
(c) His-tagged KorB NΔ30



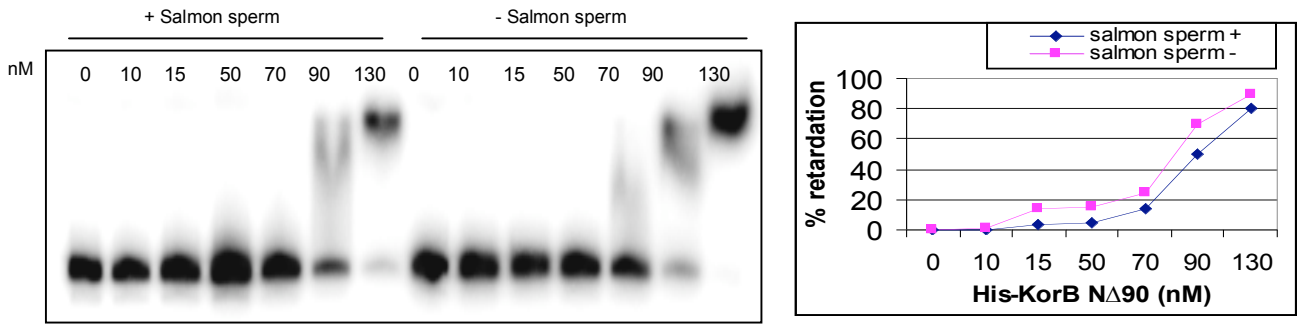


(d) Non His-tagged NΔ30 KorB

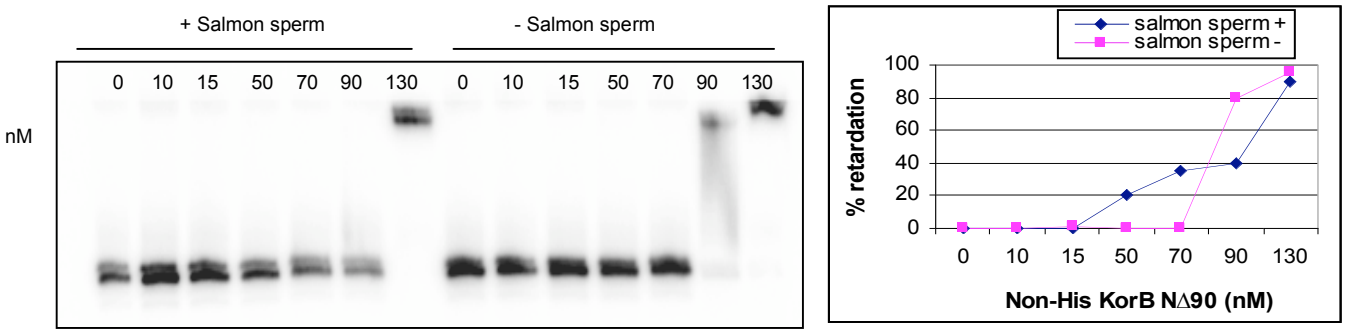




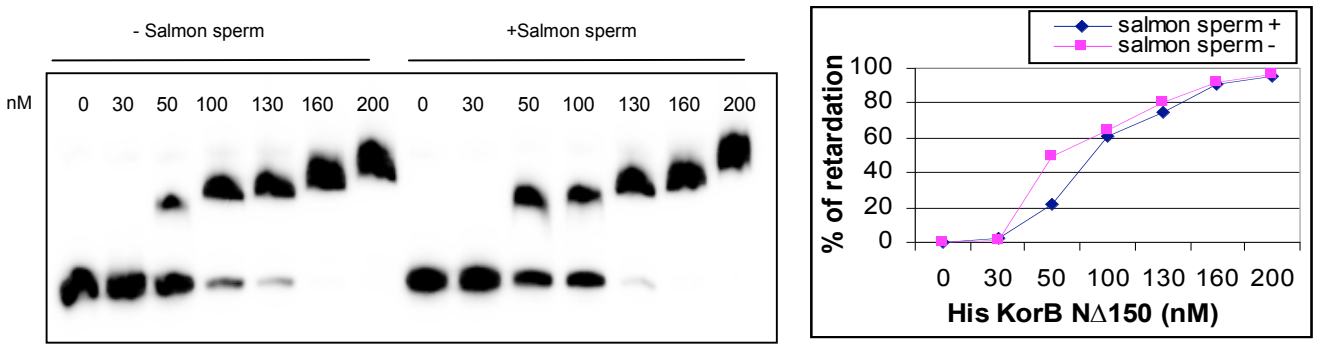
(e) His-tagged NΔ90 KorB



(f) Non His-tagged NΔ90 KorB



(g) His-tagged NΔ150 KorB



(h) Non His-tagged NΔ150 KorB

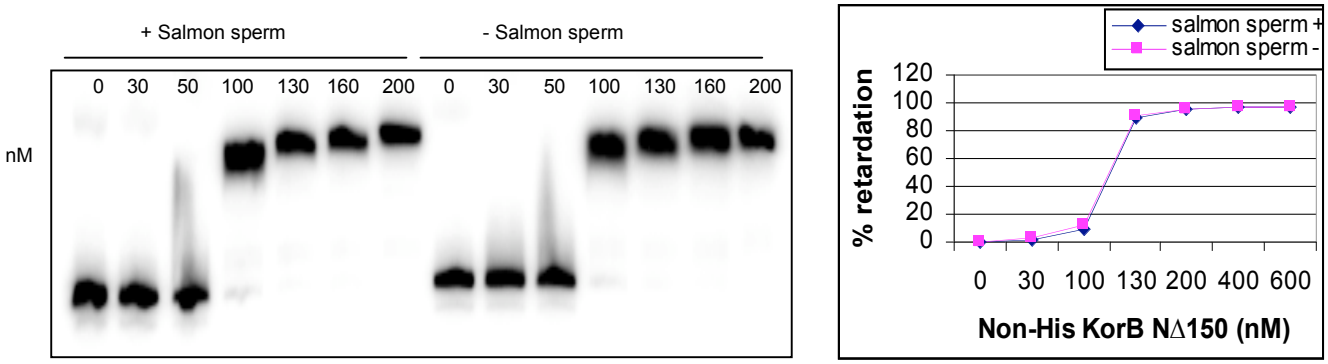


Figure 3.4: EMSAs of His-tagged and non His-tagged KorB (WT/ N-terminal deletion mutants) on the O_B operator in the presence as well as absence of competitor salmon sperm DNA. Protein concentration (in nM) used has been shown above the gel. Gel retardation data was quantified using a phosphorimager and the percentage of retarded species was plotted against KorB concentration to estimate the apparent affinity (Kapp, equal to the protein concentration giving 50% occupancy of the DNA) of the protein, as shown in graphs on the right.

3.2.4 KorB (WT and N-terminal derivatives) binding specificity to O_B operator

In order to demonstrate the binding specificity of KorB (WT and N-terminal derivatives) to the O_B containing DNA fragments EMSAs were performed with three different DNA fragments obtained by the *BsaJI* digestion of pKK113 (in which O_B is cloned between *EcoR*I – *Hind*III*),* one fragment was of different sizes i.e. 228 bp fragment containing O_B, 421 bp and 1720 bp fragments without O_B **(Figure 3.5)**. EMSA experiments using varying size fragments where only one of them has O_B suggested that KorB (WT as well as N-terminal deletion mutants) binds specifically to the 228 bp fragment containing O_B but at higher concentration they bind non-specifically to the fragments of 421 bp and 1720 bp and form high order complexes. EMSAs shown in **Figure 3.5** also indicate that KorB retard large DNA fragments at lower concentration of protein as compared to small fragments.

3.3 Discussion

Many prokaryotic regulatory proteins bind specifically to DNA with a common helix turn helix (HTH) motif. HTH is a well characterized three dimensional structure that allows many proteins to bind DNA. KorB also binds to its operator sequence 5`-TTTAGCGCG/CGCGCTAAA-3` by HTH motif (Theophilus and Thomas, 1987; Kornacki et al., 1987).

Band shifts experiments revealed that all of the KorB N-terminal deletions derivatives bind specifically to DNA fragments having O_B. The interesting finding is that the N-terminus deletion of KorB results in modulation of the strength of the interaction of the KorB with O_B. This modulation of KorB-O_B interaction by the N-terminus of KorB might be achieved in two ways. First, if WT KorB distorts DNA it needs additional energy to stress the N-terminus which could

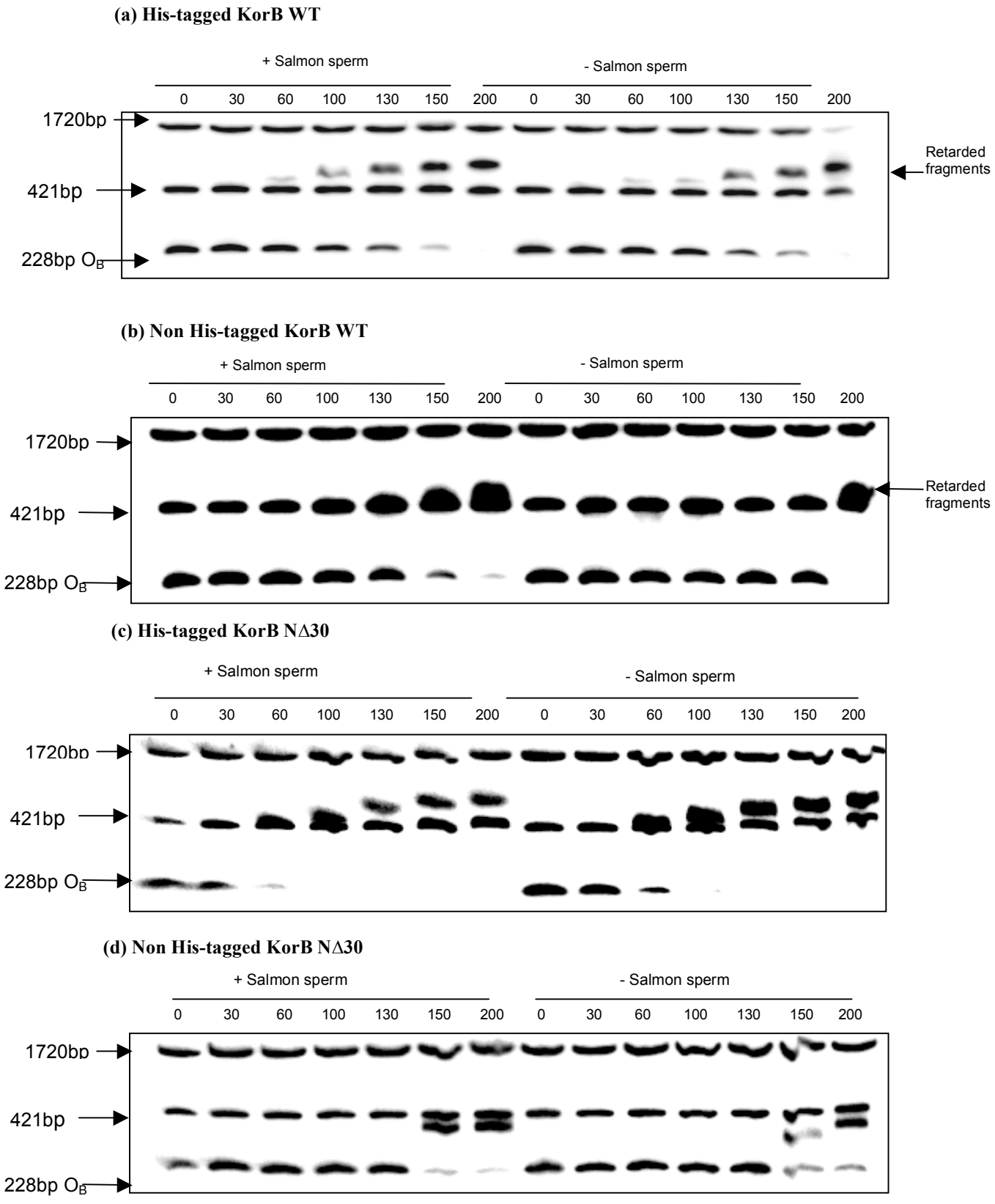


Figure 3.5: Binding specificity of His-tagged and non His-tagged WT and NΔ30 KorB to O_B operator in the presence as well as absence of competitive salmon sperm DNA. Used protein concentrations (in nM) are shown above the gel.

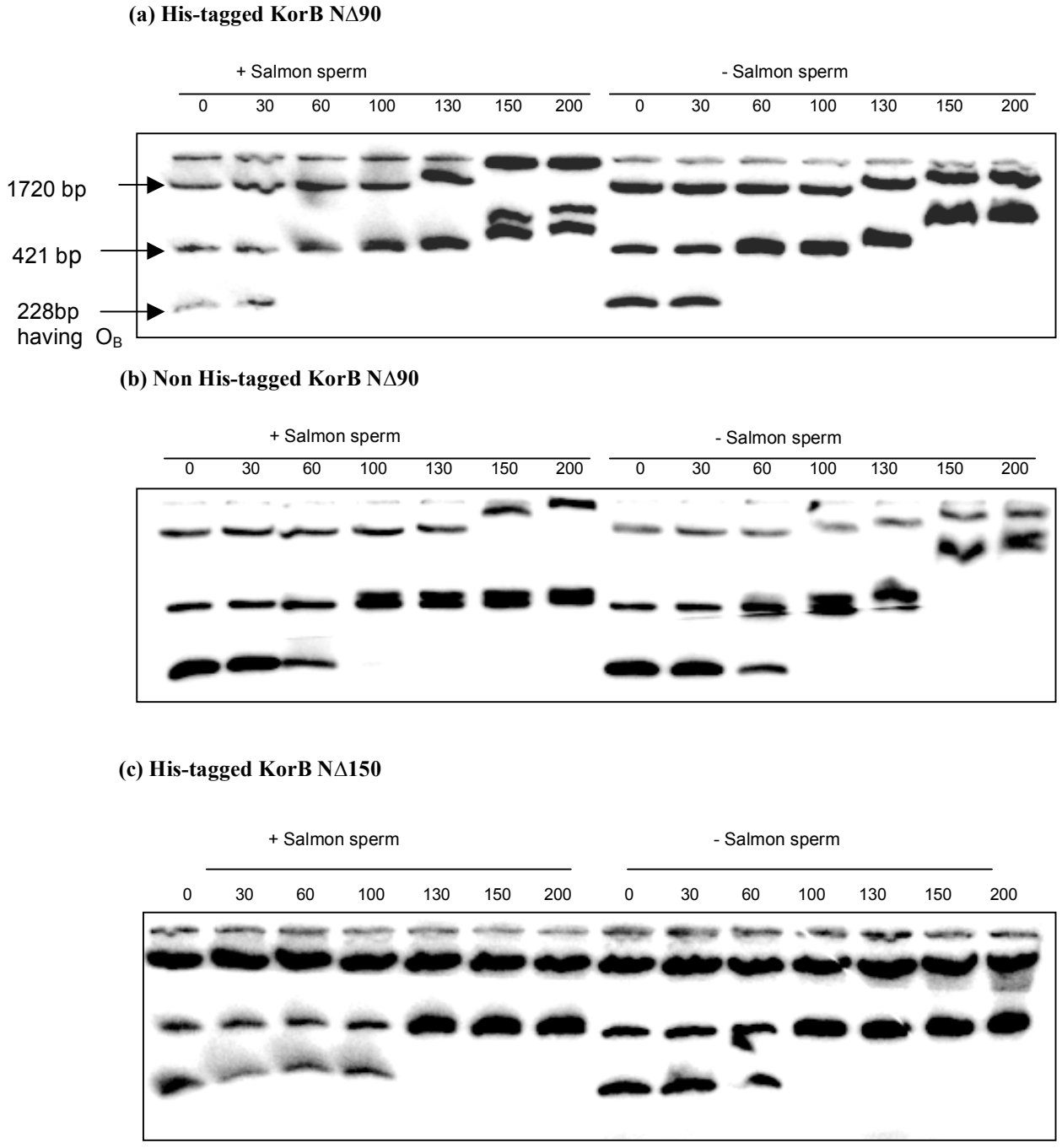


Figure 3.6: Binding specificity of His-tagged and non His-tagged NΔ90 KorB and NΔ150 KorB to O_B operator in the presence as well as absence of competitive salmon sperm DNA. Used protein concentrations (in nM) are shown above the gel.

affect the docking of KorB HTH motifs with DNA. Second, N-terminus deletion mutants of KorB could be more flexible in placing of HTH motifs in the right place at O_B site. N-terminal domain of KorB has been explored further by comparing binding affinities in the presence and absence of His-tag at the N-terminus of KorB. His-tagged KorB (WT/mutant) binds O_B slightly stronger than non His-tagged. KorB is a negatively charged protein (-21) and the presence of positively charged His-tag might increase its affinity for DNA. His-tagged proteins bind salmon sperm DNA in great amount and thus have got higher non-specific binding capacity than non His-tagged KorB proteins.

KorB binding specificity has also been determined by using three different sized DNA fragments (228 bp, 421 bp, 1720 bp) obtained by *BsaJ*I digestion of pKK113 in which O_B was cloned into *EcoR*I – *Hind*III. KorB binding site O_B was present only in the 228 bp DNA fragment while the other two fragments (421 bp and 720 bp) were used as control. Retardation gels (**Figure 3.5 and 3.6**) showed that KorB (WT/N terminal derivatives) prefers to bind the 228 bp fragment first because it has O_B in it. As the concentration of protein increases they bind to the 421 bp fragment and then the 720 bp fragment. This implies that KorB molecules bound to DNA interact with each other and can result in its bending as well as looping. This may indicate how KorB approaches and controls expression of the promoters that lie far from the KorB operators.

Chapter 4: KorB domains required for cooperativity with KorA and TrbA to regulate gene expression in RK2

4.1 Introduction

Cooperativity is the synergistic interaction between proteins, such that the effect of their combined action is greater than the product of their individual effects. Cooperative binding of repressors to DNA provides efficient regulation of transcription, which allows gene expression at low repressor concentrations, but causes rapid shutdown when repressor concentration rises above a threshold level. Examples of cooperativity in regulation include cooperativity between dimers of Lambda cI repressor protein (Ptashne, 1992) and cooperativity between *E. coli* CAP-CytR proteins to form a complex that can repress or activate transcription (Valentin-Hansen et al., 1996). In these examples, cooperativity occurs between repressor and activator proteins.

By contrast, RK2 is a very useful model to study cooperativity between repressor proteins. RK2 global regulatory proteins (i.e. KorA, KorB, TrbA and KorC) work together to establish tight control on genes involved in replication, stable inheritance and conjugative transfer **(Figure 4.1)**. KorB is a negatively charged (-21), acidic protein, consisting of 358 aa (39011 Da). It exists as a dimer in solution. KorB monomer is composed of three domains, KorB N-terminal domain (KorB-N), KorB C-terminal domain (KorB-C), KorB DNA binding domain (KorB-DBD, also called central domain), and the linker region **(Figure 4.2)**. KorB-C forms the dimerisation domain of KorB. Structural studies have shown that KorB-C has a fold which closely resembles the Src homology 3 (SH3) domain found in eukaryotic proteins and

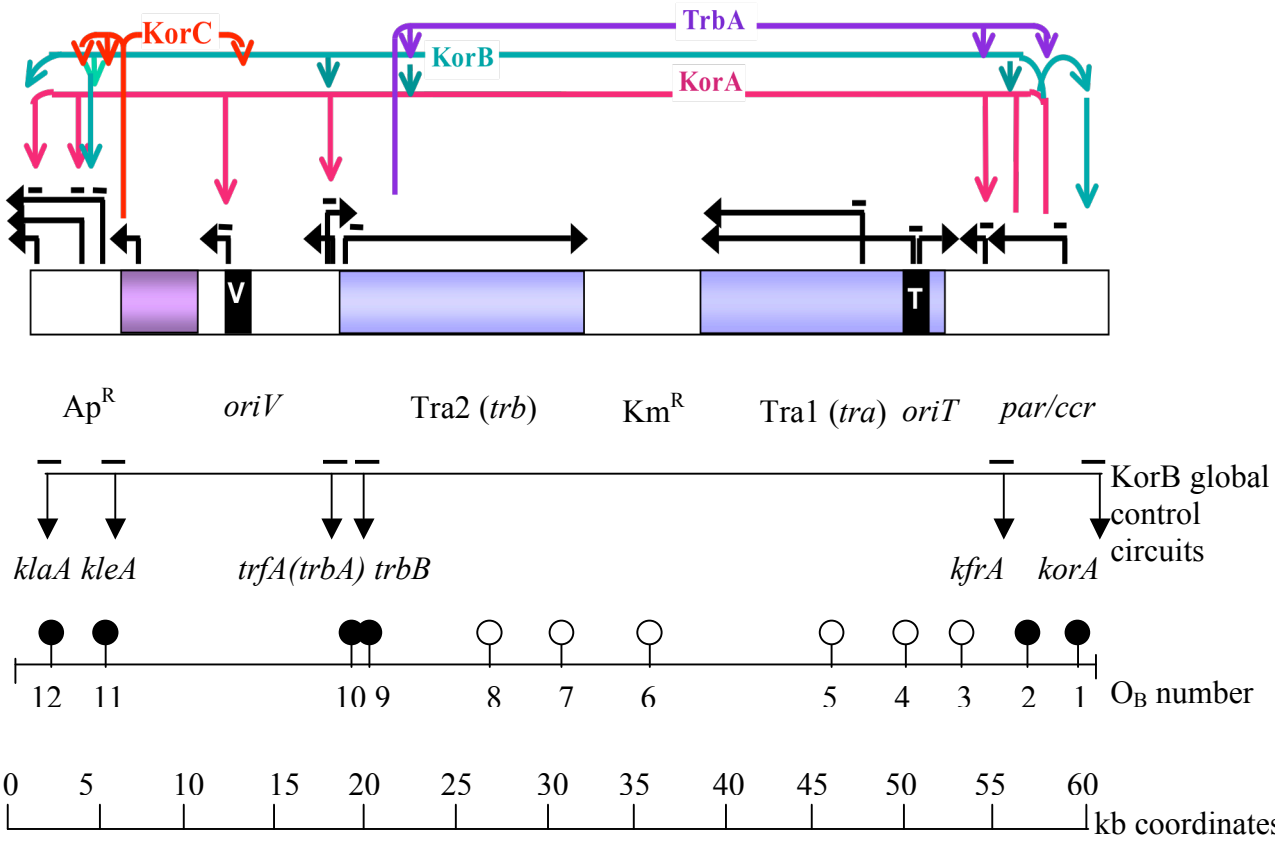


Figure 4.1: Regulation of RK2 backbone functions by KorA, KorB, TrbA and KorC. Map of RK2 showing kb coordinates, location of the KorB binding sites and the regulatory circuit they mediate. Backbone functions are shown as : V on black, vegetative replication origin; T on black, transfer replication origin; *ccr* for central control region; Tra1 a nd Tra2 are involved in the conjugative transfer of the plasmid; Amp^R is ampicilline resistance; Km^R is kanamycine resistance. KorB binding sites have been shown in white and black circles where black circles indicates sites where KorB is known to repress or repress in combination with a second global regulator, white indicates where KorB is known to bind but where a role in regulation has not been demonstrated. Horizontal arrows indicate relevant promoters. Downwards pointing arrows indicate regulatory action for a protein. At many promoters these regulatory proteins are acting together to achieve strong repression. All regulatory circuits are essentially negative (KorA, KorB, TrbA and KorC are repressors).

the HTH motif, which is required to bind DNA. The structures of KorB-C and KorB-DBD have been solved separately by Raman spectroscopy (Delbrück et al., 2002) and crystallisation (Khare et al., 2004) respectively. Structural information about KorB-N is not available, as it has not been successfully crystallised yet. KorB has a dual role: on the one hand, it is a ParB homologue and, along with IncC (ParA homologue) and O_B3, it forms the active partitioning apparatus of RK2; on the other hand, it acts as global regulatory protein which interacts with other regulators (i.e. KorA, TrbA, KorC) to control and regulate gene expression in RK2.

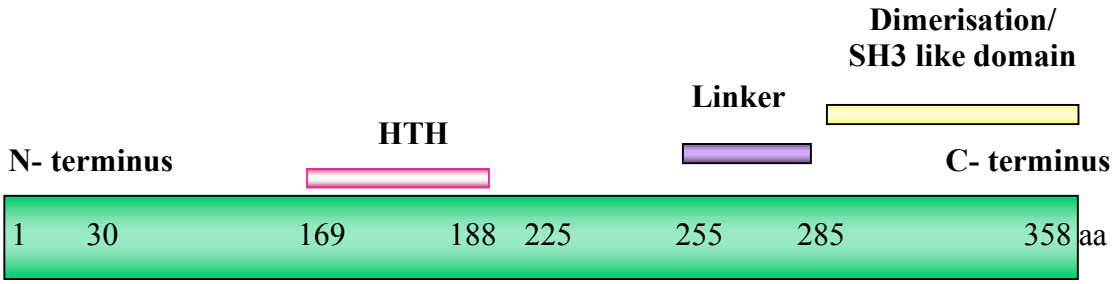


Figure 4.2: Structural and functional relationship in KorB.
KorB is 358 aa long protein. Its C-terminus (294-358 aa) is the main dimerisation domain, which structurally looks like sarc homology 3 (SH3) like domain. C-terminus (62 aa) is connected to the central domain.

KorB binds specifically to the O_B found 12 times on RK2 (Balzer et al., 1992, William et al., 1993); it forms oligomers which are detectable by chemical crosslinking (Balzer et al., 1992; William et al., 1993; Jagura-Burdzy et al., 1999b); it represses *trfAp, korAp* and *klap* when the operator is placed approximately 40 bp upstream of the transcription start point (Jagura-Burdzy et al., 1999b); it represses *kfrA, kleAp* and *trbBp* when O_B is approximately 200 bp upstream of the transcription start point (Jagura-Burdzy et al., 1999b); it represses *trbAp* when O_B is 86 bp downstream of the *tsp* (Jagura-Burdzy and Thomas, 1997).

KorB binding sites (O_{B1-12}) can be grouped by their proximity to the binding sites for either KorA or TrbA, with which KorB exhibits cooperativity (Kostelidou et al., 1999; Zatyka et al., 2001). All class I or II sites are close to sites for one or other of these second repressors: O_B1, 2, 10, 11 and 12 are close to KorA sites; O_B9 is close to a TrbA site. The other sites (class III: O_B3, O_B4, O_B5, O_B6, O_B7 and O_B8) are more than 500 bp away from a KorA or TrbA site and from promoters (Chiu et al., 2008). When KorB binds to the proximal O_B, the resultant repression and cooperativity are referred to as P-repression (proximal repression) and P-cooperativity (proximal cooperativity) respectively. For distal O_B, it is D-repression (distal repression) and D-cooperativity (distal cooperativity).

Some ParB proteins (i.e. P1 ParB, *B. subtilis* Spo0J) bind to *parS* and recruit additional ParB molecules by oligomerisation along DNA in a sequence-independent manner. This process is called spreading. Several ParB (i.e. P1 ParB and F SopB etc) proteins have also been reported to cause gene silencing when their binding site is placed near transcriptionally active genes (Lynch and Wang, 1995; Kim and Shim, 1999; Rodionov et al., 1999; Bartosic et al., 2004; Bingle at al., 2005; Dubarry et al., 2006). Gene silencing is considered to be a consequence of spreading which might be interfering locally with RNAP function or downstream in initiation of transcription (William et al., 1993; Jagura-Burdzy et al., 1999). However, Spo0J, ParB homologue of *B. subtilis,* has been shown to spread along DNA up to multiple kilo bases from its binding site, but does not cause gene silencing. KorB is not yet known for gene silencing.

In this study attempts have been made to demonstrate if KorB can spread and silence genes, and if so, which regions of KorB are required for this. Also, full-length KorB has been explored via deletion mutagenesis to reveal the domains of KorB required for cooperativity with KorA or TrbA, for proximal repression (P-repression: when O_B is close, i.e. up to 40 bp,

to the promoter) and distal repression (D-repression: when O_B is at a distance, i.e. 189 bp or more, from the promoter), and the mechanisms of repression and cooperativity.

4.2 KorB deletion mutants under study

In order to dissect KorB, deletions were made in the whole length protein ranging from N-terminus to C-terminus. Some of the KorB mutants were constructed by Kalliopi Kostelidou (NΔ30, NΔ60, NΔ90, NΔ150, Δ245-255 aa, Δ 235-555 aa, Δ255-285 aa) while C-terminal deletion mutants were constructed during this study. The method for deletion mutagenesis has been described in **Chapter 2.** Deletions made in different regions have been drawn schematically in **Figure 4.3**.

4.3 Reporter plasmids and experimental strategy

The *korA* and *trbB* promoters have been used in this study to report KorB repression and cooperativity with KorA and TrbA. *korAp* controls the expression of the central control region, which encodes KorA, IncC, KorB, KorF and KorG. *trbBp* control the expression of genes involved in the conjugative transfer of the plasmid. *korAp* is a class I promoter where O_B (KorB binding site) is located 40 bp upstream of the transcription start point (*tsp*), whereas *trbBp* is a class II promoter where O_B is located 189 bp upstream of the *tsp*. In native promoter constructs *korAp* (from which *xylE* gene was expressed to measure repression) is used to report proximal repression and *trbBp* for distal repression. It has not been clear yet if KorB uses a different mechanism for distal and proximal repression. To answer this question, new constructs of *korAp* and *trbBp* were used to report proximal as well as distal repression

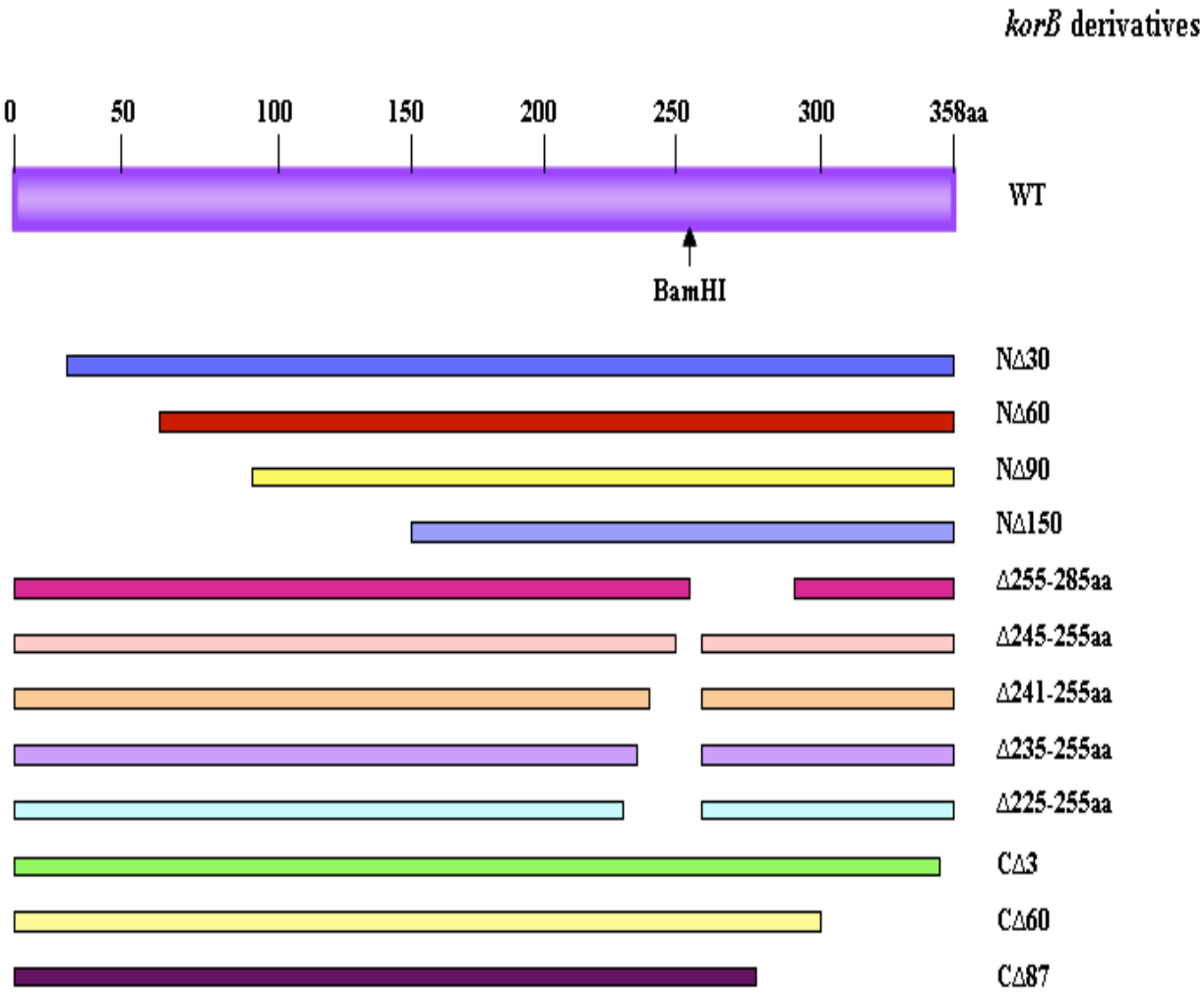


Figure 4.3: Schematic representation of the deletions made in KorB.

on both promoters (*korAp*: O_B -40 bp in pDM3.1; O_B -189 bp in pLB125, and *trbBp*: O_B -180 bp in pMZT39; O_B -46 bp in pLB40). To determine if helical position is important for KorB interaction with other proteins, reporter plasmids were used in which proximal O_B was facing the opposite face of DNA by adding 5 bp between the proximal O_B and the *trbBp* in pLB40, and then another 5 bp were added to bring back the O_B on the same face as *trbBp* (O_B -51 bp in pLB117 where O_B is facing the opposite face of the promoter; O_B -56 in pLB118 where O_B is facing on the same side as promoter). To test how far KorB can repress and cooperate with KorA and TrbA, the distance between O_B and *trbBp* in pMZT39 was increased up to 1.5 kb (O_B -278 in pLB101, O_B -636 in pLB104, and O_B-1563 in pLB105). The *korAp* and *trbBp* reporter plasmids used in this study are shown schematically in **Figures 4.4 - 4.5**, and the sequences of KorB regulated promoters in RK2, including *korAp* and *trbBp,* and the binding sites of KorB (O_B), KorA (O_A) and TrbA (O_T) are shown in **Figure 4.6.**

The *korA* and *trbB* promoters were cloned into pSC101 replicon plasmid to express the promoterless gene *xylE*, in order to measure KorB repression and cooperativity with KorA or TrbA using *xylE* assays (catechol 2, 3 oxygenase assays) as explained in **Chapter 2**. A three-vector system was created in *E.coli* (C600), which was first transformed with *korAp/trbBp* reporter plasmid and then with pMB1 replicon plasmid expressing KorB (WT/mutant) and IncQ replicon plasmid expressing KorA or TrbA. Expression of KorB, KorA and TrbA was induced from *tacp* using 0.05 mM IPTG. The whole scheme of *xylE* assays and plasmid system in *E.coli* (C600) is explained in **Figures 4.7 to 4.9**.

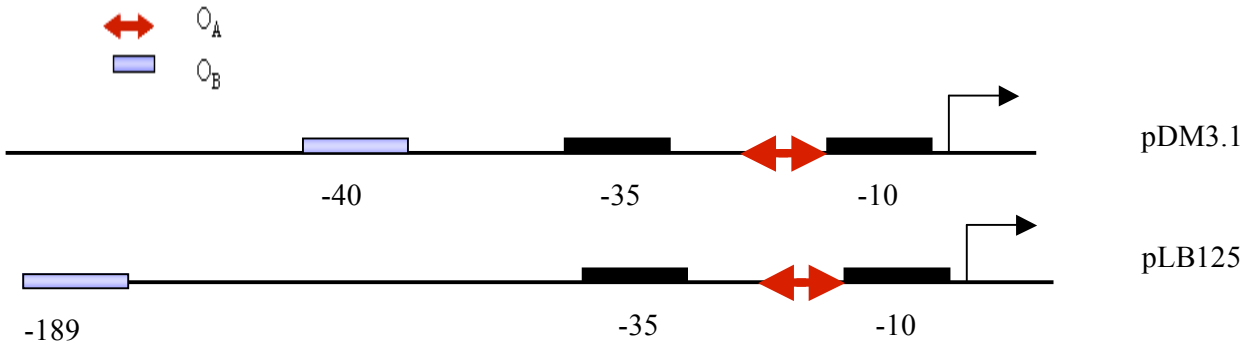


Figure 4.4: The genetic organisation of the KorB-regulated *korA* promoter constructs used in this study. From *korAp, xylE* gene was expressed to report repression and cooperativity.

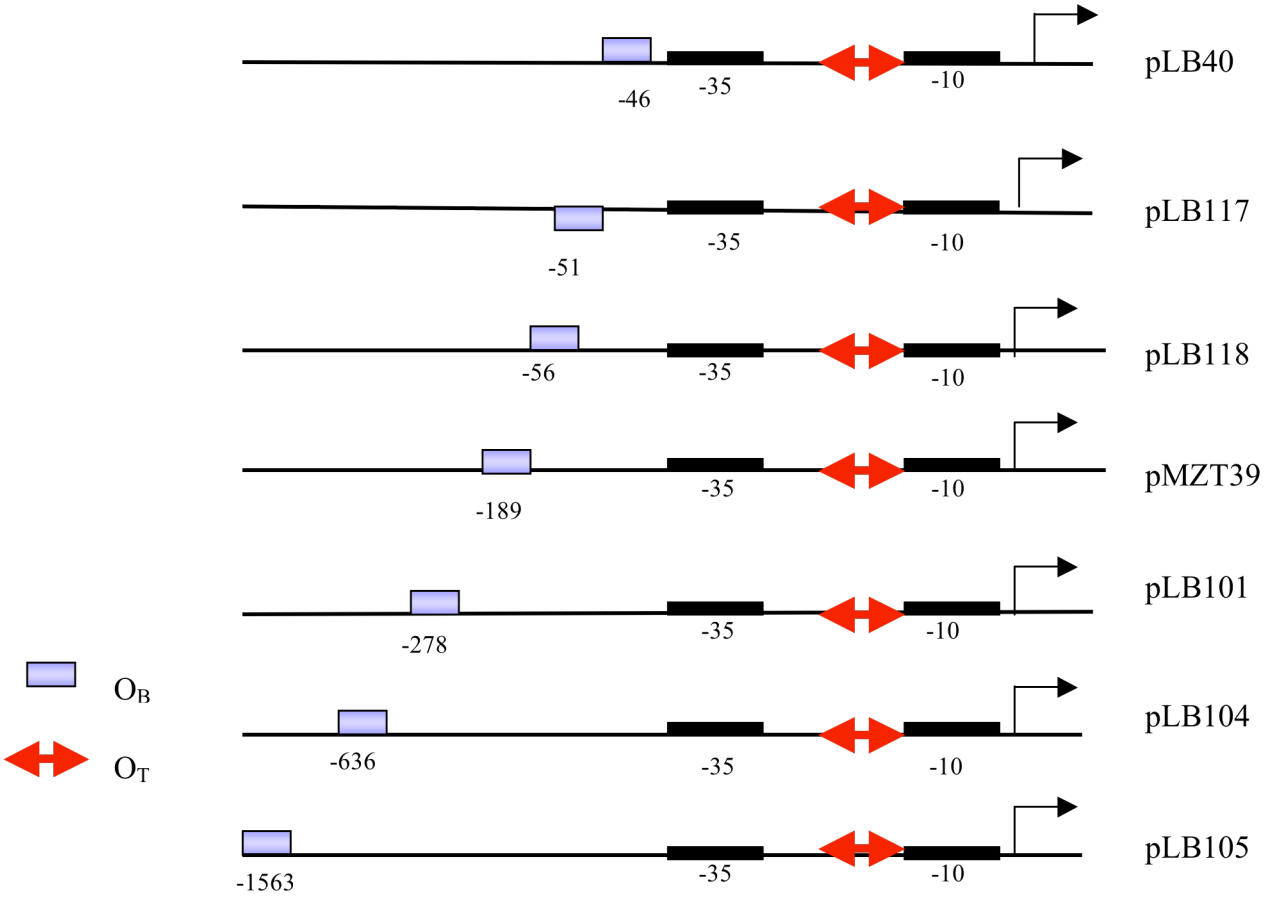


Figure 4.5: The genetic organisation of the KorB-regulated *trbB* promoter constructs used in this study. From *trbBp, xylE gene was* expressed to report repression and cooperativity.

5' - TTTAGCCGCTAAAGGTGTTGACGTGCGAGAAATGTTTAGCTAAACT-3' ***korAp***

5' - TTTAGCCGCTAAAGTTCTTGACAGCGGAACCAATGTTTAGCTAAACT-3' ***trfAp***

5' - TTTAGCCGCTAAAGTTCTTGACAGCGGAACCAATGTTTAGCTAAACC-3' ***trfAp*-1**

5' -TTTAGCCGCTAAA.....TTGACGGCTAAACACTTTCGGTATATCGT-3' ***trbBp***

5' -ATGACGTACCTCGGTGTCACGGGTATCGT- 3' ***trbAp***

Figure 4.6: The sequences of the different KorB regulated promoters. KorB binding site O_B is shown in blue, KorA binding site O_A is shown in red, and TrbA binding site O_T is shown in plum color.

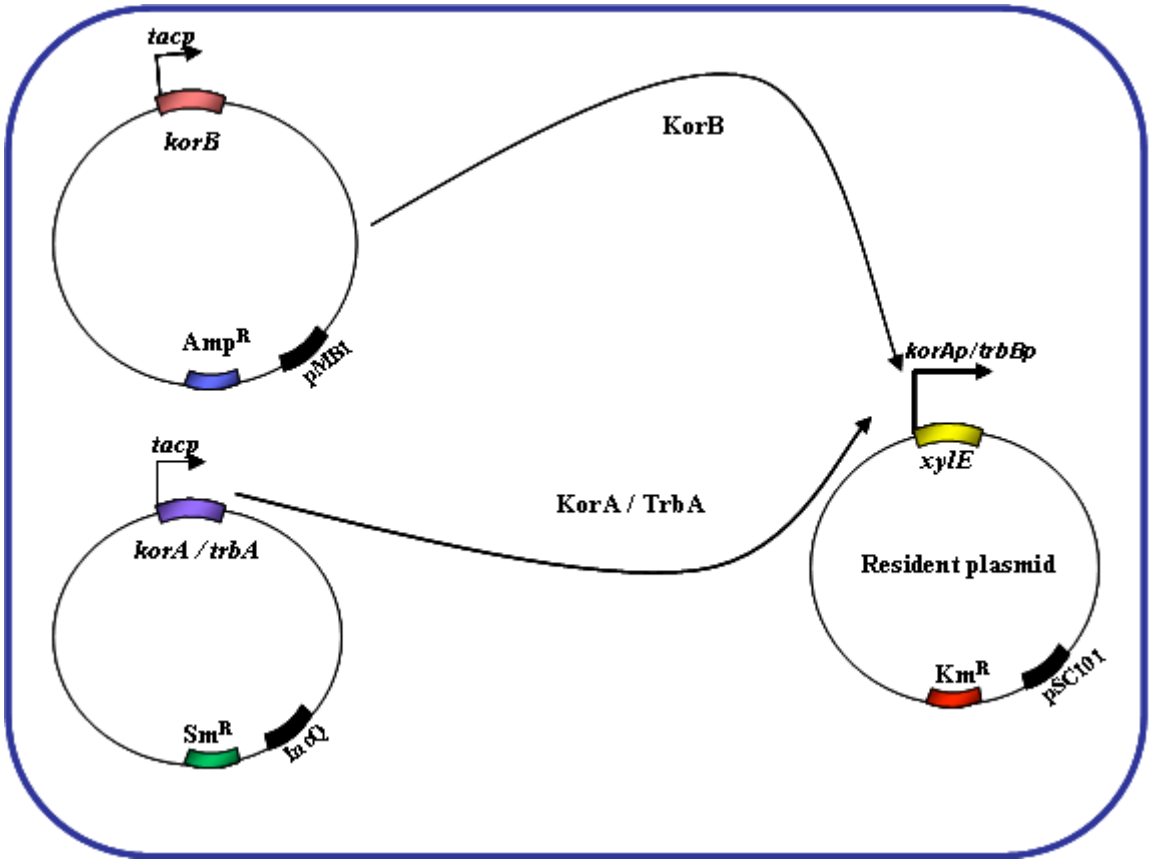


Figure 4.7: Schematic representation of the three vector system used in *E. coli* to report KorB repression and cooperativity with KorA or TrbA. Resident plasmid is pSC101 replicon, expressing *xylE* gene from the *korAp/ trbBp.* There are two *tacp* expression plasmids: one is expressing KorB, and the second is expressing either KorA or TrbA *in trans.* KorB is being expressed from high copy number pMB1 replicon and KorA or TrbA from low copy number IncQ replicon plasmid.

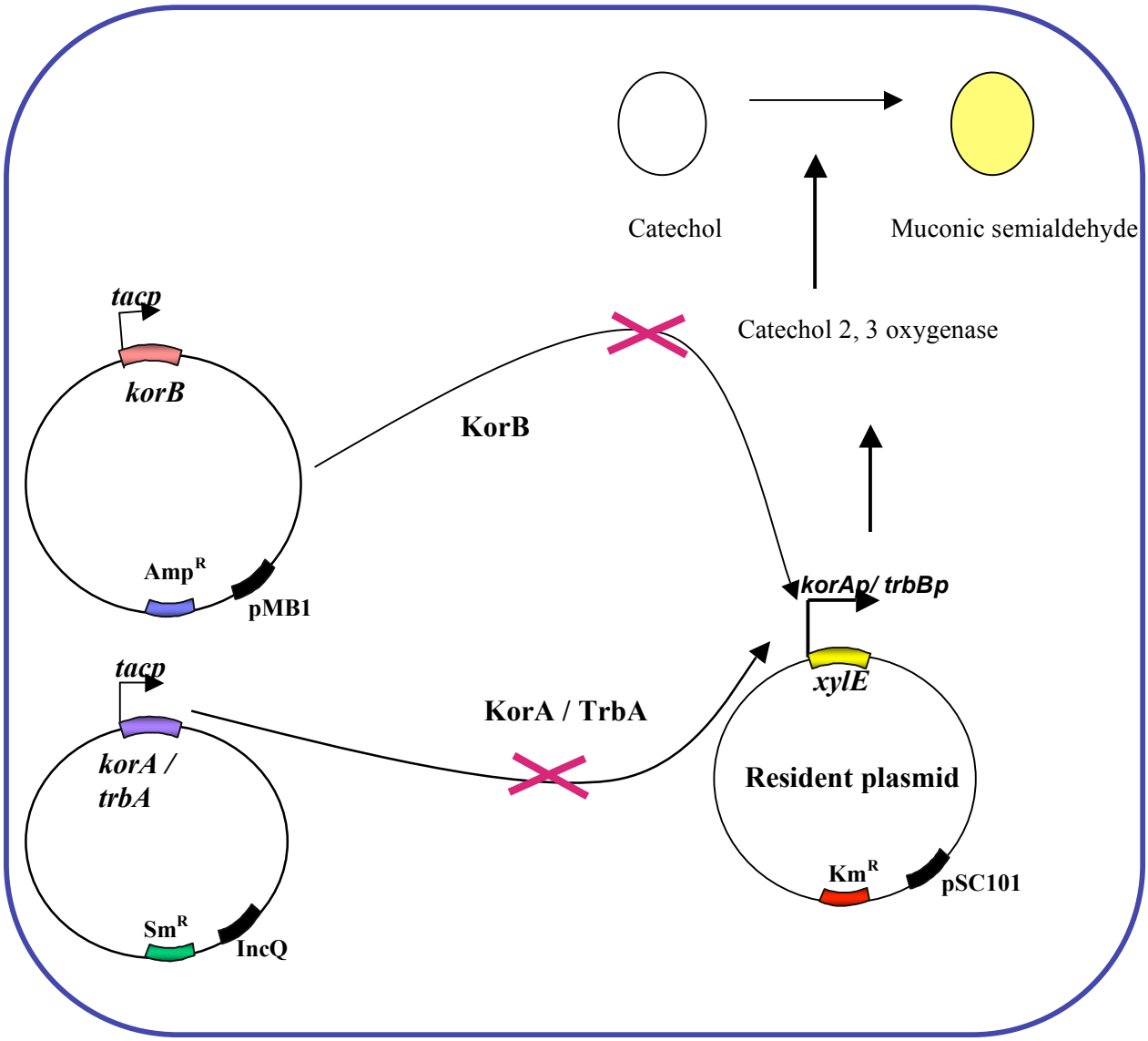


Figure 4.8: Schematic representation of *xylE* assays used to report repression and cooperativity using three vector system in *E. coli* (C600). In the absence of KorB and TrbA or KorA, *xylE* gene forms catechol 2, 3 oxygenase enzyme which converts colourless catechol into yellow coloured muconic semialdehyde. The amount of muconic semialdehyde present in the solution is measured via spectrophotometer.

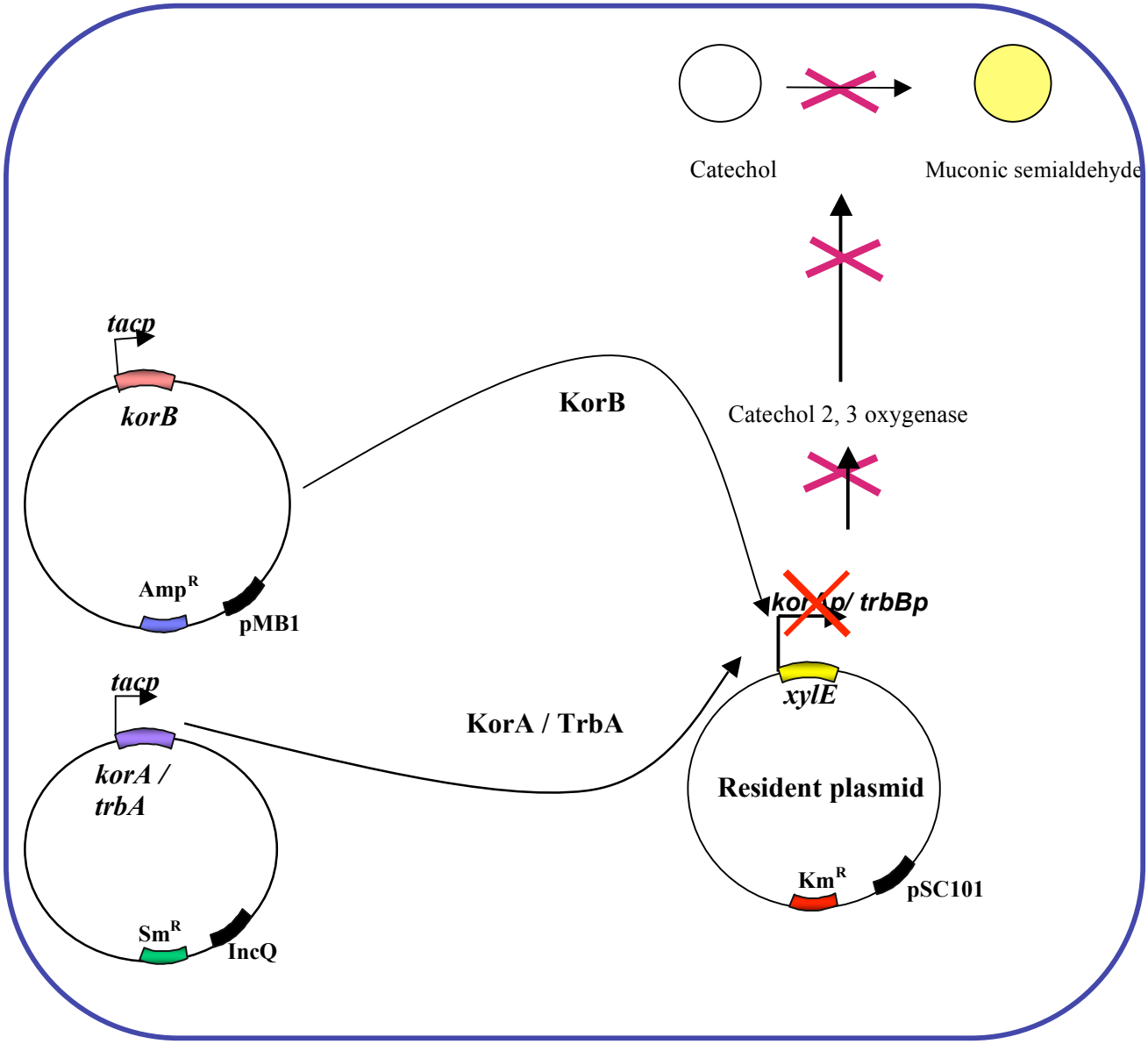


Figure 4.9: Schematic representation of reporter system for repression and cooperativity via using *xylE* assays in *E. coli* (C600). Three vectors included reporter plasmid expressing *xylE* gene from *korAp* or *trbBp* and two expression plasmids expressing KorB and either TrbA or KorA from the *tacp in trans*. Upon expression, KorB and TrbA or KorA bind to their specific binding sites close to the promoter and repress the expression of *xylE* gene from the promoter, hence there is no formation of catechol 2, 3 oxygenase enzyme and as a result there will be no muconic semialdehyde (yellow colour). This is how repression is measured spectrophotometrically by measuring the amount of muconic semi-aldehyle present in the reaction.

4.4 Results

4.4.1 KorB repression and cooperativity with KorA at *korAp*

KorB repression and cooperativity with KorA was carried out at *korAp* belonging to class I: at this promoter, the binding site for KorB is localized 40 bp upstream from the transcription start point (*tsp*) and is proximal to the promoter. Reporter plasmid pDM3.1 containing the *korAp* with proximal O_B was used to analyse repression by KorB and its cooperativity with KorA.

All KorB mutants were tested in catechol 2,3-oxygenase assays in order to check their repression and cooperativity activities. Strains were set up as presented in **Figure 4.10** so as to contain three plasmids – the reporter plasmid and two additional vectors carrying the repressor genes, i.e. *korB* and either *korA* or *trbA*, as described below. First, *E. coli* C600 cells were transformed simultaneously with two plasmids: one of these plasmids was reporter plasmid pDM3.1 (proximal O_B - *korAp* linked to the promotorless *xylE* cassette), and the other was either expression vector pDM1.1 (IncQ replicon, *tacp* empty vector), or pSTM11 (pDM1.1 plus *korA*). Second, to the *E. coli* C600 (pDM3.1, pDM1.1/ pSTM11) background was introduced either a third compatible plasmid pGBT30 (which acts as a negative control and is the vector into which the *korB* ORFs were cloned), or plasmids carrying WT or mutated *korB*. Control assays were performed using the three vector strain carrying pDM1.1 (IncQ replicon) plasmid instead of pSTM11 (KorA expression vector), which enabled us to measure the repression activity of each of the mutated KorB proteins. Expression of KorA and KorB from *tacp* was induced with 0.05 mM IPTG and assays were performed three times in triplicate as described in **Chapter 2**. Strains were set up as presented in **Figure 4.10** and the data from these assays have been recorded in **Table 4.1.**

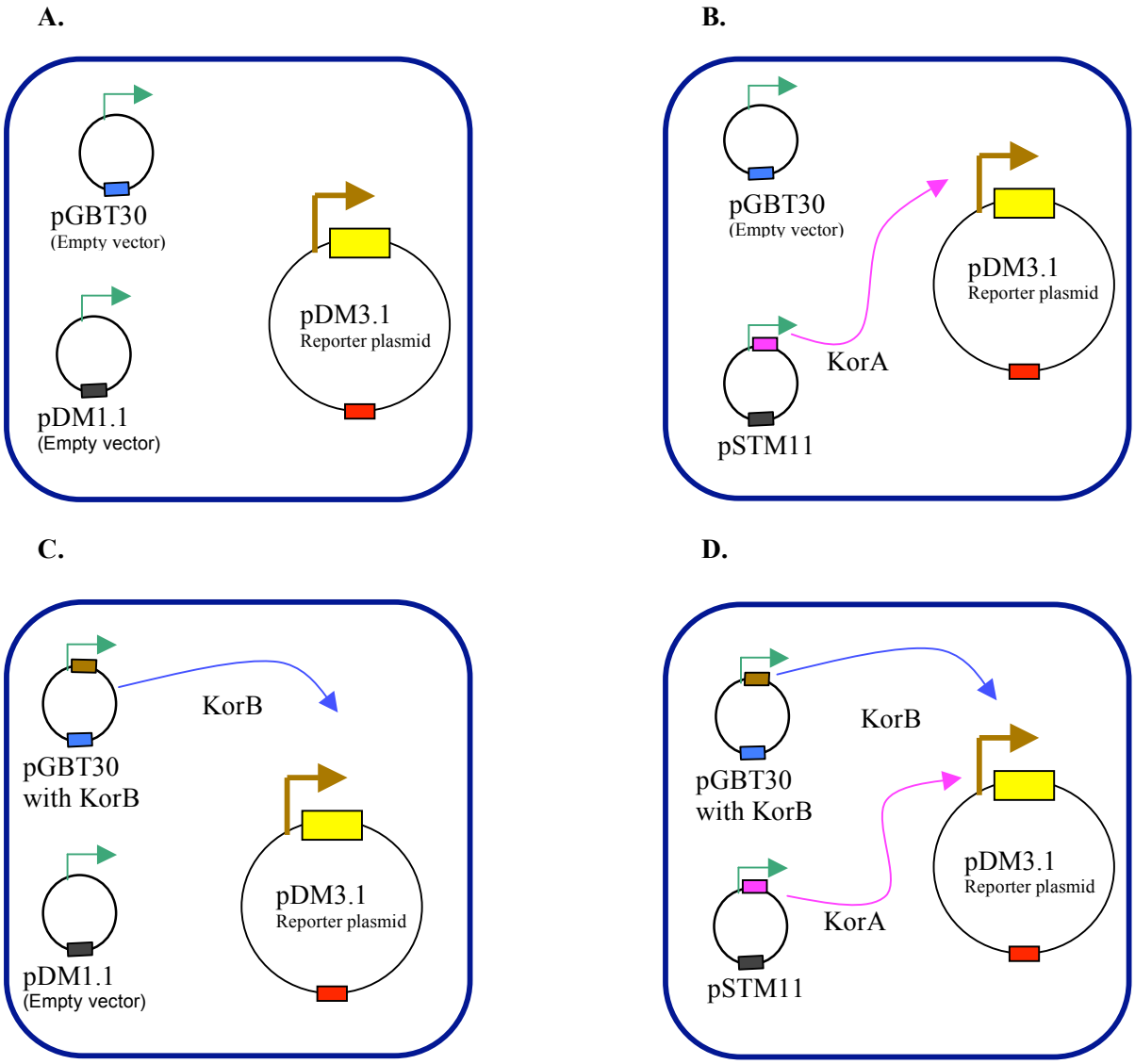
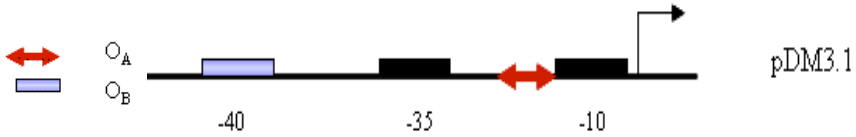


Figure 4.10: Schematic representation of *E.coli* C600 strains used to create the three vector system to report repression and cooperativity. **A**. negative control carrying pGBT30, pDM1.1 (empty IncQ replicon) and pDM3.1 (reporter plasmid *korAp-xylE*); **B.** strain which enables measurement of repressor activity by KorA alone (pSTM11 carrying *tacp-korA* instead of pDM1.1 in **A.**); **C.** strain which enables measurement of repressor activity by KorB alone (pGBT30 carrying *tacp-korB* instead of empty vector pGBT30 in **A.**); **D.** strain which enables measurement of cooperativity between KorB and KorA carrying *tacp-korB*, pSTM11 (*tacp-korA*) and pDM3.1 (*korAp-xylE*). Green arrow symbolises *tacp*; brown arrow symbolizes *korAp*; yellow box indicates *xylE*; brown box indicates *korB* and pink box indicates KorA; blue box indicates Pn cassette, red box – Km cassette and black box – Sm cassette.

Table 4.1: *In vivo* activities of *korAp* with proximal O_B in the presence of KorB (WT/ deletion mutants) and KorA.

Reporter Plasmid	**pDM3.1 (*korAp* with O_A and proximal O_B)** [A] *tsp*–O_B centre distance 40bp				
	xylE activity[x]		Repression index [R]		Cooperativity index[C]
KorB	- KorA	+KorA	KorB Ri_B	KorB + KorA Ri_{AB}	Ci
Empty vector	0.97	0.099	-	9.7	-
WT	0.003	0.000018	334	53889	17
Δ245-255 aa	1.27	0.47	0.76	2.06	0.28
Δ241-255 aa	1.03	0.21	0.94	4.85	0.5
Δ235-255 aa	1.15	0.42	0.85	2.3	0.28
Δ255-285 aa	0.0047	0.000048	206	20208	10
NΔ30	0.0032	0.00002	303	48500	16
NΔ60	0.0062	0.000079	156	12278	8
NΔ90	0.0048	0.000047	202	20638	10
NΔ150	0.0053	0.000069	183	14058	8
CΔ3	0.05	0.00032	19	2982	16
CΔ60	0.048	0.00021	20	4515	23
CΔ87	0.067	0.00045	14	2170	16

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\text{xylE activity - KorA and –KorB}}{\text{xylE activity + KorA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{AB}\text{ (KorA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_{AB}\text{ (KorA+ empty vector)}}$

As can be seen from **Table 4.1**, KorB mutants with deletions in the region 225 to 255 aa (i.e. KorB Δ245-255 aa, Δ241-255 aa, Δ235-255 aa) have lost their ability to repress from proximal O_B at *korAp*. Their repression activity is doubled in the presence of KorA, which could be only the effect of KorA as their cooperativity index value is very low (i.e. Ci is less than 0.5). However, deletion of the linker region Δ255-285 aa makes KorB a weak repressor compared to WT, but it is still functional with cooperativity index 1.7 times less than KorB WT.

Deletion of 30 aa, 60 aa, 90 aa and 150 aa from the N-terminus did not affect the ability of KorB to repress and cooperate with KorA from proximal O_B. Particularly KorB NΔ30 repression indexes (Ri_B 303 and Ri_{AB} 48500) are quite close to that of WT (Ri_B 334 and Ri_{AB} 53888), and cooperativity indexes differ by only one as Ci for WT is 17 and for NΔ30 is 16.

KorB C-terminal deletions made it monomeric protein. All of the C-terminal deletions (i.e. CΔ3, CΔ60 and CΔ87) showed repression (Ri_B 19, 20 and 14 respectively) weaker than WT and N-terminal deletions, however, strong repression was recovered in the presence of KorA with cooperativity index values (Ci=16, 23 and 16) much closer to KorB WT (Ci=17).

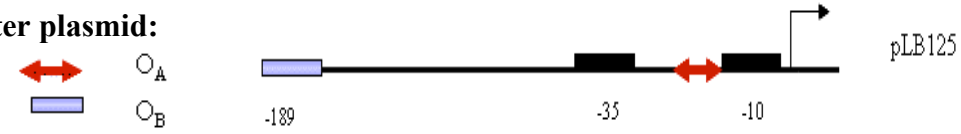
4.4.2 Repression and cooperativity between KorB and KorA at modified *korAp*

KorB mutants (Δ255-285 aa, NΔ30, NΔ60, NΔ90, NΔ150, CΔ3, CΔ60 and CΔ87) which were able to repress and cooperate with KorA at native *korAp* with proximal O_B, were chosen to test if they are still able to repress modified *korAp*, interact with RNAP and cooperate with KorA while placing the O_B at a distance. For this experiment, a distal O_B (-189 bp) *korAp* construct linked to *xylE* (pLB125) was used as a reporter plasmid, which has been described in section 4.3 of this Chapter. Once again assays of catechol 2,3-oxygenase were performed

Table 4.2: *In vivo* activities of modified *korAp* with distal O_B in the presence of KorB (WT/ deletion mutants) and KorA.

Reporter Plasmid	**pLB125 (*korAp* with O_A and distal O_B)** [A] *tsp*–O_B centre distance 189bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	- KorA	+KorA	KorB Ri_B	KorB + KorA Ri_{AB}	Ci
Empty vector	1.1	0.1	-	11	-
WT	0.04	0.00033	28	3333	11
Δ255-285aa	0.45	0.0016	2.4	688	26
NΔ30	0.62	0.0021	1.8	524	27
NΔ60	0.74	0.026	1.5	42	3
NΔ90	0.66	0.015	1.7	73	4
NΔ150	0.79	0.019	1.4	58	4
CΔ3	1.2	0.2	0.92	5.5	0.54
CΔ60	0.96	0.13	1.2	8.6	0.68
CΔ87	1.4	0.42	0.8	2.6	0.3

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE} \text{ activity - KorA and –KorB}}{\textit{xylE} \text{ activity + KorA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{AB} \text{ (KorA + KorB)}}{Ri_B \text{ (KorB WT/mutant + empty vector)} \times Ri_{AB} \text{ (KorA+ empty vector)}}$

using a three-vector system. However this time, instead of pDM3.1 carrying RK2 *korAp,* pLB125 containing *korAp* region with O_B1 cloned not 40 bp upstream of the promoter but 189 bp was used. Otherwise the system was created as described previously. The results of these assays are presented in **Table 4.2**.

Table 4.2 shows that KorB lacking the linker region (Δ255-285 aa) and NΔ30 lose repression (Ri_B 0.45 and 0.62 respectively) on their own from distal O_B, which is restored in the presence of KorA with repression index values Ri_{AB} 688 and 527 respectively. Their cooperativity index values are almost same i.e. Ci is 26 for Δ255-285 aa and 27 for NΔ30. The rest of the N-terminal mutations (i.e. NΔ60, NΔ90, NΔ150) showed weak repression and cooperativity with cooperativity index values (Ci) 3, 4 and 4 respectively. However, KorB C-terminal mutations (i.e. CΔ3, CΔ60 and CΔ87) completely lose the ability to show distal repression, and having KorA doesn't make any difference on their repression index values. Their Ri_B values are less than 1.5; Ri_{AB} values are less than 10, and Ci values are less than 1 again. These values are very low compared to WT (Ri_B=28, Ri_{AB}=3333, Ci=11).

4.4.3 Repression and cooperativity between KorB and TrbA at *trbBp*

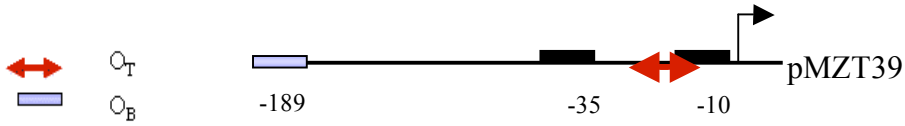
As a next step, repression and cooperativity were analysed between KorB mutants and another RK2 regulator, TrbA, in the regulation of the *trbBp*. Native *trbBp* has O_B at a distance of 189 bp and TrbA binding site O_T overlapping the -10 region of the promoter. *trbBp* provides a case where we can study KorB interaction with another global regulator (TrbA) at a distance, and screen KorB mutants defective in distal repression. All of the KorB deletion mutants were chosen to test for distal repression and cooperativity with TrbA at *trbBp*.

Catechol 2, 3 oxygenase assays were performed using the three vector system. The three strains were set up as described previously in section 4.4.1.1 and also shown in **Figure 4.10**,

Table 4.3: *In vivo* activities of *trbBp* with distal O_B in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pMZT39 (*trbBp* with O_T and distal O_B)** [A] *tsp*–O_B centre distance 189bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	KorB	KorB + TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci
Empty vector	1.2	0.8	-	1.5	-
WT	0.032	0.002	38	600	10
Δ245-255aa	1.1	0.78	1	1.5	0.94
Δ241-255aa	1	0.75	1.2	1.6	0.88
Δ235-255aa	0.9	0.69	1.3	1.7	0.87
Δ255-285aa	0.46	0.0043	2.6	279	71
NΔ30	0.41	0.0045	2.9	266	60
NΔ60	0.92	0.48	1.3	2.5	1.3
NΔ90	0.85	0.31	1.4	3.9	1.8
NΔ150	0.92	0.52	1.3	2.3	1.2
CΔ3	1.4	0.94	0.86	1.28	0.99
CΔ60	1.3	0.89	0.9	1.35	0.97
CΔ87	1.5	0.86	0.8	1.4	1.2

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

with plasmid pMZT39 (carrying *trbBp* linked to promotorless *xylE* cassette) used instead of pDM3.1 or pLB125. Assays were performed as described in Chapter 2 and data is presented in **Table 4.3**.

Table 4.3 shows that KorB internal deletion mutants (i.e. Δ245-255, Δ241-255, Δ235-255 aa) were completely defective in repression and cooperativity (Ri_B values 1, 1.2, and 1.3), and the presence of TrbA doesn't make any difference on their repression abilities i.e. Ri_{TB} values 1.5, 1.6 and 1.7 respectively. Their cooperativity index (Ci) is also very low and less than 1. However, KorB linker deletion mutant Δ255-285 and N-terminal mutant NΔ30 lose D-repression (Ri_B values 0.46 and 0.41), which is restored in the presence of TrbA with Ri_{TB} values 0.0043 and 0.0045. KorB NΔ30 and TrbA cooperate with Ci value 6 times higher than KorB Δ255-285. The rest of the N-terminal mutants (i.e. NΔ60, NΔ90, NΔ150) lose distal repression with Ri_B values 0.92, 0.85 and 0.92 but show slight difference in the presence of TrbA with Ri_{TB} values 2.5, 3.9 and 2.3 respectively and cooperativity index values less than 2. All of the C-terminal mutants i.e. CΔ3, CΔ60 and CΔ87 were completely defective in repression and cooperativity, similarly to deletion mutants within 235-255 aa. Their cooperativity index (Ci) was also less than 1.5.

These observations suggest that an increase in repressor activity is due to cooperative interaction with TrbA. It appears that cooperativity does not always depend on the ability to repress. This study has also identified deletion mutants, i.e. NΔ30 and Δ255-285 aa, which were affected in distal repression but had very strong interaction with TrbA.

4.4.4 Repression and cooperativity between KorB and TrbA at modified *trbBp*

Repression activities of all KorB deletion mutants were measured at modified *trbBp* where O_B was placed proximally to the promoter at -46 bp, in order to check if repression and

cooperativity can be restored in repression defective KorB mutants (i.e C-terminal mutants) simply by changing the localisation of O_B and if indeed the result observed at *trbBp* was due to a change in interactions with RNAP. However it was possible that localisation of the O_B and the big distance between KorB binding site and O_T influenced cooperative interaction between TrbA and mutated KorB proteins at *trbBp*. Once again assays of catechol 2,3-oxygenase were performed using a three-vector system. Reporter plasmid *xylE* gene was expressed from modified *trbBp* with O_B cloned at 40 bp (pLB40) upstream instead of 189 bp (pMZT39). Otherwise system was created as described previously. The results of these assays are presented in **Table 4.4**.

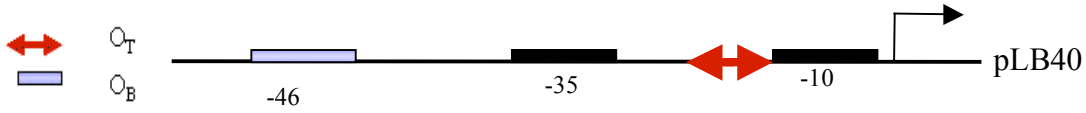
Table 4.4 shows that KorB internal deletion mutants (i.e. Δ245-255, Δ241-255, Δ235-255 aa) are completely defective in repression and cooperativity at *trbBp* irrespective of the proximity of O_B to the promoter. Their Ci values are less than 1 which is very low compared to WT (Ci=35). However all of the N-terminal mutants (i.e. NΔ60, NΔ90, NΔ150) showed strong repression and cooperativity with Ri_B values 281, 167 and 184; Ri_{TB} values 13432, 3600 and 4500; and Ci values 28, 13 and 14, respectively. KorB linker region mutant Δ255-285 aa also showed strong repression and cooperativity with Ri_B=450, Ri_{TB}=5789 and Ci=20, compared to WT Ri_B=500, Ri_{TB}=30000 and Ci=35. KorB C-terminal mutants repressed more weakly than N-terminal mutants but showed strong cooperativity, with Ri_B values 60, 75, 64; Ri_{TB} = 1800, 2571 and 1184; and Ci=18, 20 and 11, respectively.

Once again, KorB internal deletion mutants (i.e. Δ245-255, Δ241-255, Δ235-255 aa) showed loss of repression and cooperativity which was not influenced either by distance between O_B and O_T or changes in interactions between KorB and RNAP bound at the promoter. It implies that these KorB mutants lack the important regions required to function as a repressor and to interact with TrbA. KorB C-terminal mutants are weak repressors but show cooperativity with

Table 4.4: *In vivo* activities of modified *trbBp* with proximal O_B in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pLB40 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 45bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	No TrbA	TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci^4
Empty vector	0.9	0.53		1.7	
WT	0.0018	0.00003	500	30000	35
Δ245-255aa	1.1	0.8	0.8	1.1	0.8
Δ241-255aa	0.99	0.7	0.9	1.29	0.8
Δ235-255aa	1.4	1.1	0.6	0.82	0.7
Δ255-285aa	0.002	0.000057	450	15789	20
NΔ30	0.0032	0.000067	281	13432	28
NΔ60	0.0054	0.00025	167	3600	13
NΔ90	0.0049	0.0002	184	4500	14
NΔ150	0.0053	0.00031	170	2903	10
CΔ3	0.015	0.0005	60	1800	18
CΔ60	0.012	0.00035	75	2571	20
CΔ87	0.014	0.00076	64	1184	11

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

TrbA from proximal O_B, which shows that KorB needs to be dimeric to repress strongly, but in presence of TrbA it might opt a different conformation and expose other regions which might be interacting with RNAP.

4.4.5 Effect of the helical position of O_B on KorB repression and cooperativity with TrbA

As a next step, experiments were performed to test if KorB mutants improve repression and cooperativity with TrbA when helical position of O_B is changed proximally to *trbBp*. For this purpose, reporter plasmid was used with modified *trbBp* with O_B cloned at -51 bp (pLB117) and -56 bp (pLB118). These promoter constructs have been explained in detail in section 4.3 of this Chapter.

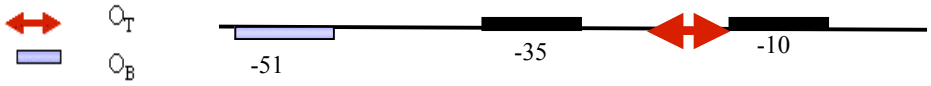
Once again catechol 2,3-oxygenase assays were performed using a three vector system as described previously. The results of these assays are presented in **Table 4.5** and **4.6**.

Table 4.5 and **4.6** show that changing the helical position does not make any difference on the repression and cooperativity by KorB mutants. Repression and cooperativity indexes values do not differ much from data obtained with pLB40-*trbBp* with O_B cloned at -40 as described in the previous section. This shows that KorB is very flexible in repression. However, this flexibility in repression and cooperativity should also be tested by changing the helical position of distal O_B.

Table 4.5: *In vivo* activities of modified *trbBp* with proximal O_B facing the opposite face of the promoter in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pLB117 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 50bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	No TrbA	TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci^4
Empty vector	1.03	0.57		1.8	
WT	0.002	0.000027	515	38148	41
Δ245-255aa	0.75	0.32	1.4	3.2	1.3
Δ241-255aa	1.2	0.7	0.9	1.5	0.92
Δ235-255aa	0.83	0.43	1.2	2.4	1.06
Δ255-285aa	0.0033	0.000045	312	22888	41
NΔ30	0.0016	0.000031	812	41935	29
NΔ60	0.0069	0.00025	188	5200	15
NΔ90	0.0085	0.00021	153	6190	22
NΔ150	0.0074	0.00026	176	5000	16
CΔ3	0.018	0.00041	57	2512	24
CΔ60	0.016	0.0003	64	3433	30
CΔ87	0.017	0.0007	61	1471	13

A **Reporter plasmid:**



pLB117

X Values of *xylE* activities from which all these repression indexes have been calculated.

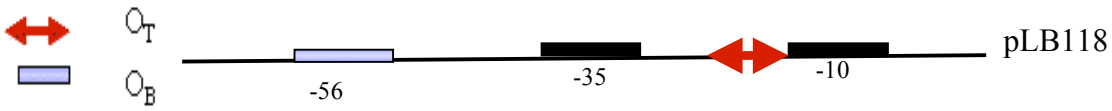
R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

Table 4.6: *In vivo* activities of *trbBp* with proximal O_B facing the same face of the promoter (reporter plasmid constructed by adding 5bp between O_B and O_T in pLB117) in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pLB118 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 55bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	No TrbA	TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci[4]
Empty vector	0.96	0.65		1.5	
WT	0.0022	0.000025	436	38400	59
Δ245-255aa	0.67	0.5	1.4	1.9	0.9
Δ241-255aa	0.86	0.63	1.1	1.5	0.94
Δ235-255aa	0.75	0.49	1.3	2	1
Δ255-285aa	0.0033	0.000048	290	20000	46
NΔ30	0.0059	0.000067	163	14328	60
NΔ60	0.0078	0.00024	123	4000	22
NΔ90	0.0084	0.000298	114	3221	19
NΔ150	0.0096	0.00039	100	2461	17
CΔ3	0.018	0.00047	53	2042	26
CΔ60	0.017	0.00052	56	1846	22
CΔ87	0.02	0.00072	48	1333	18

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

4.4.6 KorB (NΔ30 and Δ255-285aa) can repress through cooperativity with TrbA even when O_B is located approximately 1.5 kb away from the promoter

KorB mutants NΔ30 and Δ255-285 aa, which were able to show D-repression only in the presence of KorA or TrbA where O_B is 189 bp away from the promoter, were chosen to test if they are still repressing and cooperating with KorA/TrbA when O_B site is moved further away up to 1.5 kb.

Catechol 2,3-oxygenase assays were performed to report repression and cooperativity using a three vector system in *E. coli* C600. However, this time, the reporter plasmid contained *trbBp* region with O_B9 -278 bp (pLB101) or -636 bp (pLB104) or -1563 bp (pLB105) instead of -189 bp (pMZT39) or -40 bp (pLB40). Otherwise the system was created as described previously. The results of these assays are presented in **Table 4.7.**

Table 4.7 shows that repression by both of KorB mutants (i.e. NΔ30 and Δ255-285 aa) acting on their own was largely unaffected by changes in O_B9-*trbBp* distance (there was a bit of decrease in KorB repression at an O_B–*tsp* distance of 1563 bp). The combined repression by KorB NΔ30 or KorB Δ255-285 aa and TrbA was still significant, with Ri_{TB} values 63 and 45, and cooperativity index values 52 and 44 respectively at the greatest O_B–O_T distance tested (1563 bp, pLB105).

Table 4.7: *In vivo* activities of *trbBp* with distal O_B (distance between O_B and *tsp* is more than 200 bp) in the presence of KorB and TrbA. (a) xylE activity (b) repression index

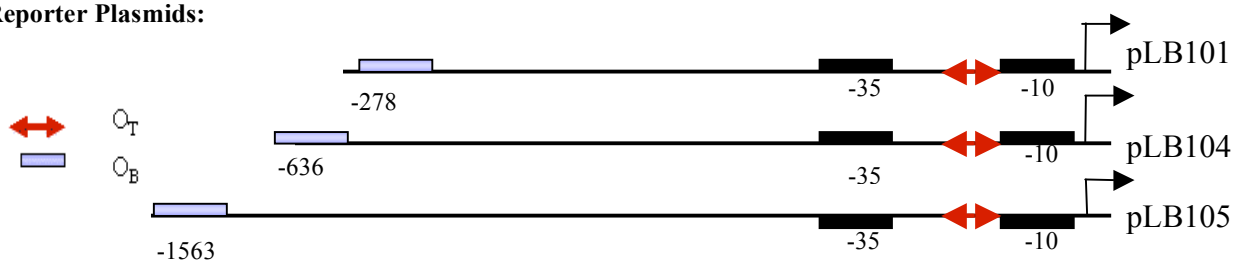
A.

Reporter plasmids (tsp–O_B centre distance)	**pLB101 [A] (278 bp)**		**pLB104 [A] (636 bp)**		**pLB105 [A] (1563bp)**	
	xylE activity[x]		*xylE* activity[x]		*xylE* activity[x]	
KorB	KorB	KorB + TrbA	KorB	KorB + TrbA	KorB	KorB + TrbA
Empty vector	0.9	0.68	0.89	0.72	1	0.9
WT	0.022	0.0019	0.024	0.0019	0.045	0.0037
NΔ30	0.44	0.0073	0.67	0.0087	0.75	0.016
Δ255-285 aa	0.59	0.0068	0.75	0.0073	0.87	0.022

B.

Reporter Plasmids	**pLB101 [A]**			**pLB104 [A]**			**pLB105 [A]**		
korB derivatives *in trans*	**Repression index[R]**		**Ci**	**Repression index[R]**		**Ci**	**Repression index[R]**		**Ci**
	KorB Ri_B	KorB+TrbA Ri_{TB}		KorB Ri_B	KorB+TrbA Ri_{TB}		KorB Ri_B	KorB+Trb Ri_{TB}	
Empty vect.	-	1.3	-	-	1.2	-	-	1.1	-
WT	41	474	9	37	468	11	22	270	14
NΔ30	2	123	46	1.3	102	64	1.33	63	52
Δ255-285	1.5	132	67	1.2	122	86	1.15	45	44

A Reporter Plasmids:



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

4.4.7 KorB gene silencing

Transcriptional silencing is a very interesting phenomenon that occurs by binding of the regulatory proteins to *cis*-acting sites that are called silencers as they mediate silencing by bi-directional inactivation of gene expression. During gene silencing, DNA forms a structure that prevents the interaction of sequence-specific DNA-binding proteins with their cognate sequences, and thus gene expression is blocked (Rine, 1999).

To determine how interaction with an adjacent protein may influence KorB recruitment on the DNA, we used the general observation that ParB proteins are known to silence genetic functions at a distance (Lynch and Wang, 1995). Since silencing has not formally been reported for KorB, this study provides the first confirmation that expression of KorB *in trans* to a pSC101 replicon plasmid carrying an O_B site can cause plasmid loss.

Gene silencing assays also required a three vector system in *E. coli* C600. *E. coli* C600 cells were first transformed with two plasmids: a resident plasmid (pGBT72 or pGBT73, as shown in **Figure 4.11**) and either an empty vector (pDM1.1, *tacp* and IncQ replicon plasmid; it is a negative control vector in which KorA and TrbA ORFs have been cloned), or a *korA* expression vector (pSTM11; *tacp*, IncQ replicon plasmid), or a *trbA* expression vector (pLB25; *tacp,* IncQ replicon plasmid) to get ***E. coli* C600 (resident plasmid: pGBT72 or pGBT73) (pDM1.1-empty vector/ pSTM11-*korA* / *trbA* expression vector)**. Secondly, *E. coli* C600 (resident plasmid) (empty vector/ *korA* / *trbA* expression vector) was transformed again to introduce a third compatible plasmid pGBT30 (which is a negative control and is the vector into which the *korB* ORFs were cloned) or plasmids carrying WT or mutated *korB* to get ***E. coli* C600 (resident plasmid: pGBT72 or pGBT73) (pDM1.1-empty vector/ pSTM11-*korA* / *trbA* expression vector) (pGBT30 -empty vector/KorB, WT or mutant,**

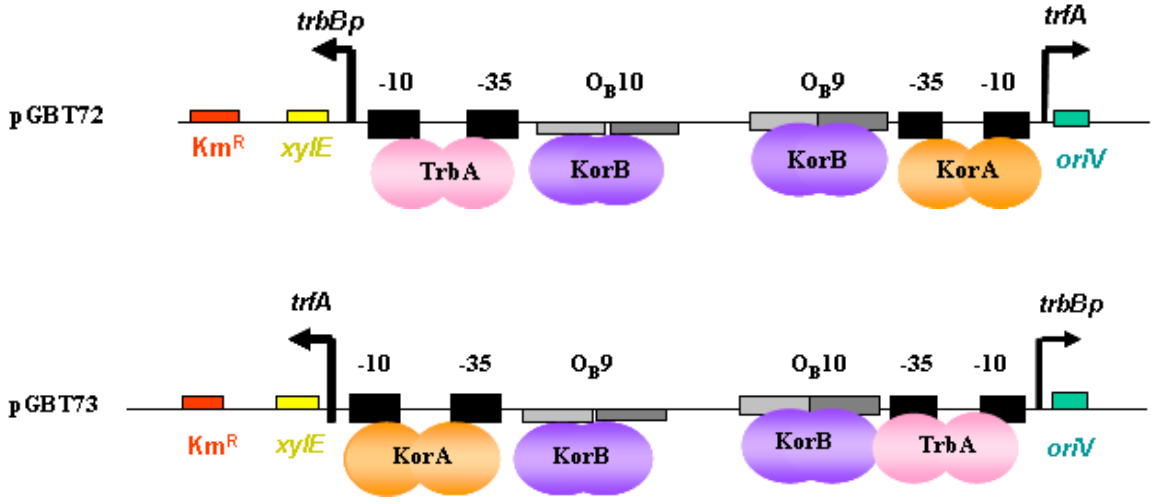


Figure 4.11: Genetic organisation of pGBT72 and pGBT72 which were used in gene silencing

Table 4.8: Gene silencing of KorB in the presence and absence of KorA and TrbA. KorB and TrbA/KorA were expressed *in trans* from *tacp* plasmids in *E.coli* C600 (pGBT72)/ (pGBT73).

Resident plasmids used for gene silencing	**pGBT72**			**pGBT73**		
	Regions inserted in pSC101-based plasmids (pGBT72 and pGBT73) and concentration of IPTG needed to induce silencing[b]					
IPTG in mM	**O_B *trfAp* O_A > < O_T *trbBp* O_B**			**O_B *trbBp* O_T > <O_A *trfAp* O_B**		
Repressors present *in trans*[a]	KorB	KorB + KorA	KorB + TrbA	KorB	KorB + KorA	KorB + TrbA
Empty Vector	-	-	-	-	-	-
KorB WT	0.5	0.05	0.05	0.5	0.05	0.05

- = No silencing
0.5/ 0.05= mM IPTG at which gene silencing was achieved

a. Repressor genes under the control of the tac promoter were provided from the following plasmids: pMMV811 (KorB), pSTM11 (KorA), pLB25 (TrbA)
b. Gene silencing was assessed as described in Chapter 2.

expression vectors). *E. coli* C600 strains having three vectors were selected on LBA plates supplemented with the selection of all of the three plasmids and with varying concentration of IPTG (i.e. 0, 0.01, 0.05, 0.5 and 1 mM IPTG) to induce the expression of KorA and KorB from *tacp*. Strains were set up as presented in **Figure 4.12**. The data from these assays are presented in **Table 4.8**.

The target pSC101-based resident plasmids (i.e. pGBT72 and pGBT 73) used here contained a region spanning the *trfA* and *trbB* promoters that fire outwards from the fragment. Each promoter has an O_B site upstream, O_{B10} for *trfAp* and O_{B9} for *trbBp*, but also each has either a KorA binding site or a TrbA binding site between the O_B site and the end of the fragment. The only difference between the plasmids was the orientation of the *trfAp*/*trbBp* fragment. KorB expression caused loss of the plasmid, irrespective of the orientation of the inserted fragment **(Table 4.8)**. As stated in the Introduction, KorA and TrbA can bind close to and interact cooperatively with KorB (Kostelidou et al., 1999; Zatyka et al., 2001). If silencing is due to simple spreading of KorB along the DNA, then binding of KorA or TrbA between the KorB binding site and the gene it is silencing should block this silencing. Thus if KorA or TrbA can act as a road-block in this experiment, expression of KorA or TrbA along with KorB should block the silencing effect in one orientation but not the other. The results showed that on the contrary, no orientation effect was observed. In fact the second protein reduced the level of IPTG needed to see a significant silencing effect, irrespective of whether or not the binding site for the second protein was between O_B and the pSC101 replicon. Neither KorA nor TrbA caused silencing alone. These results raise questions about exactly how KorB silences in this test. Whether or not it is by spreading, handcuffing of plasmid DNA molecules (McEachern et al., 1989) or some other mechanism, KorA and TrbA clearly do not block the process but rather appear to promote the recruitment of KorB. This strengthens the possibility that cooperativity with KorA and TrbA may result in higher

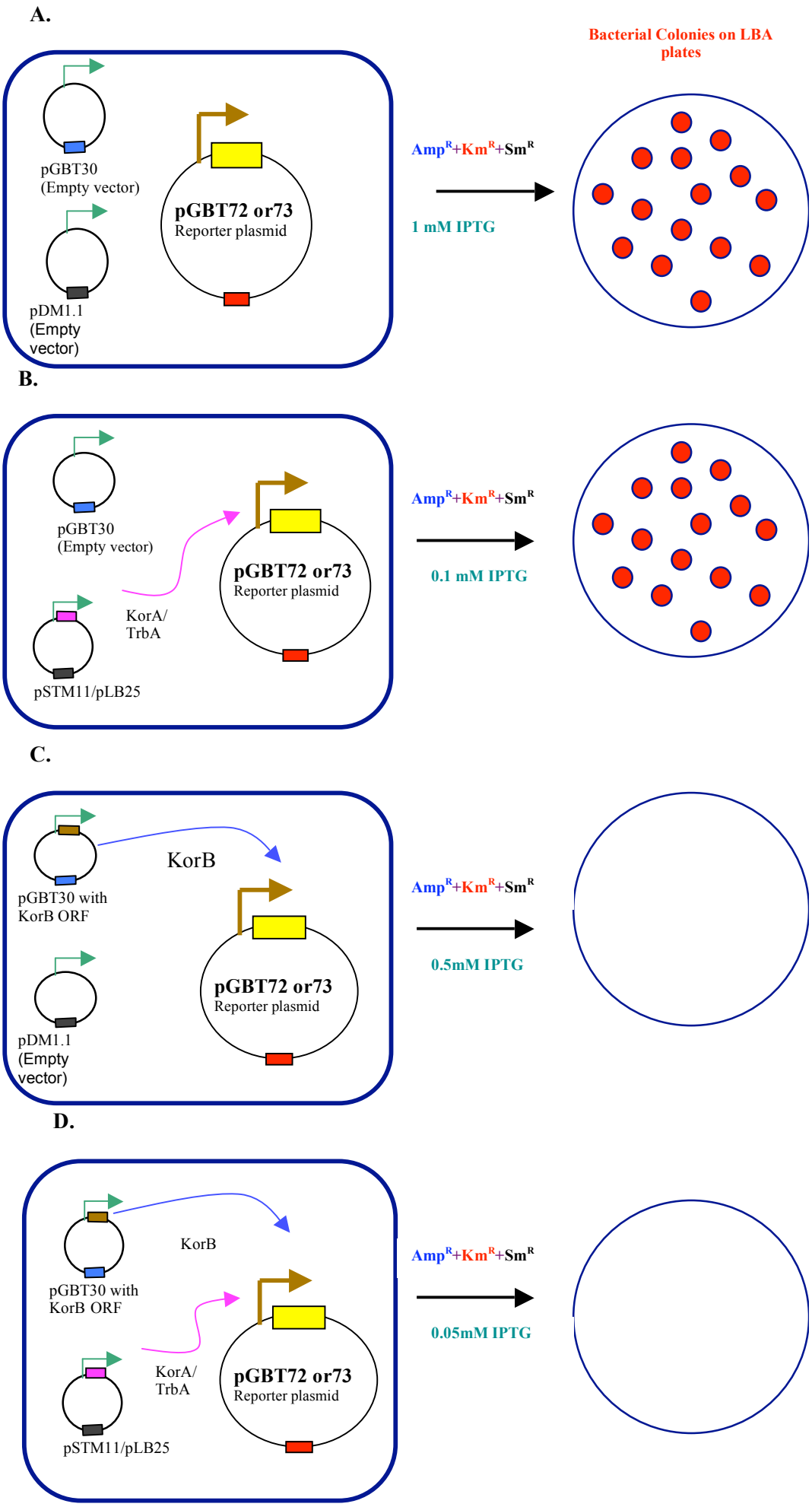


Figure 4.12: Schematic representation of the three vector system in *E.coli* C600 to report gene silencing by KorB and its potentiation by KorA and TrbA. This figure is also summarising the results obtained with KorB WT. *E.coli* C600 cells carrying three plasmids were selected on LBA plates with the selection of all of the three plasmids and different concentrations of IPTG. The concentration of IPTG required for the loss of colonies has been mentioned under arrows. Highest concentration of IPTG used was 1 mM.

A. *E. coli* C600 strain, acting as a negative control, is carrying resident plasmid (pGBT72 or pGBT73) and empty vectors (pGBT30, negative control for KorB expression vector, and pDM1.1, negative control for KorA/TrbA expression vector);

B. *E. coli* C600 strain, which enables measurement of the effect of KorA or TrbA alone on gene silencing, is carrying an empty vector (pGBT30) and KorA or TrbA expression vector *(*pSTM11 and pLB25 respectively)

C. *E. coli* C600 strain, which enables measurement of gene silencing activity by KorB alone (pGBT30 carrying *tacp-korB* instead of empty pGBT30 in **A.**);

D. *E. coli* C600 strain, which enables measurement of he potentiation of KorB gene silencing by KorA/TrbA , is resident plasmid and expression vectors for KorB and KorA/TrbA.

Green arrow symbolises *tacp* brown arrow symbolizes *korAp*; yellow box indicates *xylE*; brown box indicates *korB* and pink box indicates KorA; blue box indicates Pn cassette, red box – Km cassette and black box – Sm cassette.

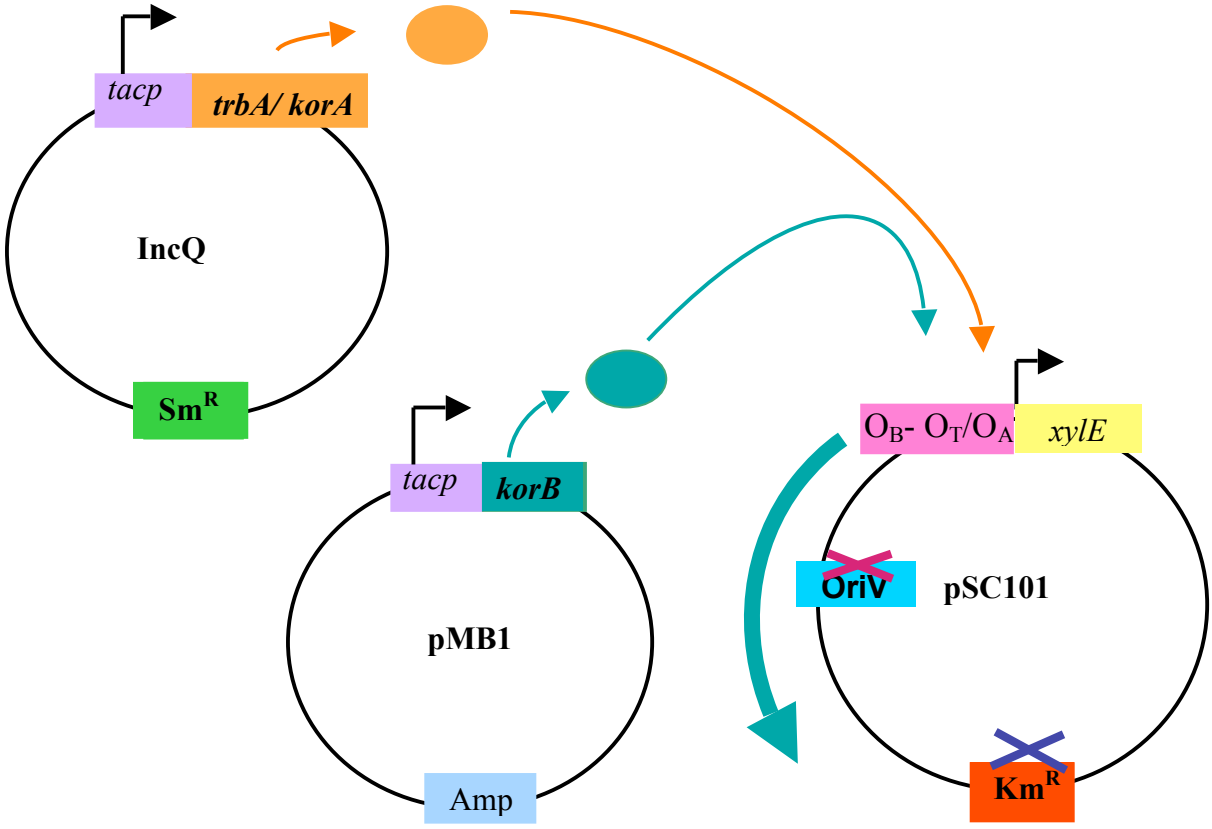


Figure 4.13: Mechanism of gene silencing by KorB. KorB binds to O_B and spread along DNA irrespective of the direction and thus interferes with the expression of nearby genes i.e. pSC101 replicon and Km^R in the above plasmid. This is how KorB would not be allowing the pSC101 replicon to replicate and Km^R gene to express itself and hence resulting in the loss of pSC101 replicon plasmid. KorB can achieve same results with less amount of protein when KorA and TrbA are present *in trans*. It could be that KorB binds better to DNA in the presence of KorA and TrbA. This is not clear yet how KorA or TrbA potentiate gene silencing by KorB. It could be that KorA/TrbA sit next to KorB and helps it to spread over long distances or KorA/TrbA might make complex with KorB first which has higher affinity to bind and spread along DNA and causing gene silencing by wrapping the DNA around itself or by interfering with RNAP.

occupancy of specific regions of the plasmid and disfavour binding at sites where this cooperativity does not occur.

4.4.8 KorB domains required for gene silencing

KorB deletions mutants which were able to bind DNA were chosen to test for their ability for gene silencing. Strains were set up as shown in **Figure 4.12** and described in previous section and results have been presented in **Table 4.9**.

Most of the KorB mutants which were able to bind DNA were defective in gene silencing. This implies that DNA binding is not sufficient for KorB to silence genes. KorB NΔ30 and Δ255-285 showed gene silencing at higher concentration of IPTG (i.e. gene silencing observed at 1 mM IPTG when alone and 0.5 mM IPTG when a second global regulatory protein was present) than what is required to observe gene silencing for KorB WT (0.5 mM IPTG when KorB is alone and 0.05 mM IPTG when a second global regulatory protein is present). Any further mutations in N-terminus and internal region resulted in proteins defective in silencing genes. KorA or TrbA potentiates gene silencing by NΔ30 and Δ255-285 but they had no effect on the KorB mutants defective in gene silencing (i.e. Δ235-255 aa, Δ241-255 aa, Δ245-255 aa, NΔ60, NΔ90, NΔ150, CΔ3, CΔ60, CΔ87)

Table 4.9: Gene silencing by KorB (WT/mutants) in the presence and absence of KorA and TrbA. KorB and TrbA/KorA were expressed *in trans* from *tacp* plasmids in *E.coli* C600

Resident plasmids used for gene silencing assays	**pGBT72/pGBT73**		
KorB	IPTG (mM) required to observe gene silencing		
Repressors present *in trans*[a]	KorB	KorB + KorA	KorB + TrbA
Empty Vector	-	-	-
KorB WT	0.5	0.05	0.05
NΔ30	1	0.1	0.1
NΔ60	-	-	-
NΔ90	-	-	-
NΔ150	-	-	-
Δ255-285aa	1	0.1	0.1
Δ245-255aa	-	-	-
Δ230-255aa	-	-	-
CΔ3	-	-	-
CΔ60	-	-	-
CΔ87	-	-	-

- = No silencing
1, 0.1, 0.5 and 0.05= IPTG concentration in mM at which gene silencing was achieved

c. Repressor genes under the control of the tac promoter were provided from the following plasmids: pMMV811 (KorB), pSTM11 (KorA), pLB25 (TrbA)

d. Gene silencing was assessed as described in Chapter 2.

4.5 Discussion

KorB is a global regulatory protein that binds DNA and represses genes depending upon its oligomeric state (Motallebi-Veshareh et al., 1992). In this study attempts have been made to narrow down the regions of KorB required for cooperativity with KorA and TrbA to regulate gene silencing, repression from proximal and distal O_B at *korAp* and *trbBp*.

Table 4.10: KorB (WT/deletion mutant) repression at proximal and distal O_Bs

KorB	Proximal O_B (P- O_B)		Distal O_B (D- O_B)	
	P-repression	**P-cooperativity**	**D-repression**	**D-cooperativity**
WT	Y	Y	Y	Y
NΔ30	Y	Y	x	Y
NΔ60	Y	Y	x	x
NΔ90	Y	Y	x	x
NΔ150	Y	Y	x	x
Δ255-285	Y	Y	x	Y
Δ241-255	x	x	x	x
Δ245-255	x	x	x	x
Δ235-255	x	x	x	x
CΔ3	Y	Y	x	x
CΔ60	Y	Y	x	x
CΔ87	Y	Y	x	x

Y= repression
x= no repression

Regions required for D-repression= Full length KorB
Regions required for D-cooperativity = all domains except 30 aa from N-terminal and linker region (255-285 aa)
Regions required for P-repression and P-cooperativity= 235-255 aa

* All of the KorB derivatives reported here are able to bind specifically to DNA having O_B.

All of the N-terminal deletions in KorB resulted in the loss of its ability to repress at a distance (D-repression) which was restored by second repressor (KorA *korAp* and TrbA at

trbBp) fully only in the case of NΔ30. However weak combined repression (KorA + KorB or TrbA + KorB) was observed in the rest of the KorB N-terminal mutants (i.e. NΔ60, NΔ90, NΔ150). NMR has also confirmed that NΔ150 can interact with KorA (Rajeskar et al., unpublished). KorB-N is required for D-repression but is not essential for P-repression and cooperativity with KorA and TrbA. These results, along with previous ones (Jagura-Burdzy et al., 1999; Lukaszewicz et al., 2002) show that full length KorB is essential for its long range repression and gene silencing, in contrast to tasks from proximal O_B i.e. P-repression and P-cooperativity.

Deletion within KorB region 235-255 aa results in polypeptides which can bind DNA but are fully defective in transcriptional repression, irrespective of the localisation of the binding site (O_B) from the promoter and the presence of KorA/TrbA. KorB lacking the linker region (255-285 aa) is affected in D-repression whereas P-repression and cooperativity (distal as well as proximal) remains undisturbed. This suggests DNA binding alone is not sufficient for repression even from proximal O_Bs, implying that direct interaction with RNAP and TrbA/KorA might be necessary. KorB region 235-255 aa should be explored further by substitution mutagenesis to determine a single amino acid responsible for D- and P-repression and cooperativity with KorA and TrbA.

Deletion of only three amino acids from KorB-C results in a monomeric protein as observed by glutaraldehyde crosslinking and analytical ultracentrifugation (data not shown). KorB-C mutants were able to bind DNA and show P-repression and P-cooperativity but defective in D-repression, which could not be restored even in the presence of KorA or TrbA. This indicates that KorB-C -terminal is essential to achieve D-repression and D-cooperativity whereas P-repression remains unaffected. These results are consistent with Lukaszewicz et al., 2002, who also showed that KorB-C is responsible for dimerisation of the protein and

deletions in this domain resulted in loss of downstream and distal repression (when O_B is placed 86 bp downstream of *tsp* and up to 189 bp upstream of *tsp,* respectively), whereas 101 aa from C-terminus can be removed without complete loss of proximal repression (when O_B is placed 40 bp upstream of the *tsp*). NMR studies shows that CΔ60 had no effect on the KorB-KorA complex (Rajasekar et al., unpublished). It suggests that KorB-C mutants might be defective in spreading along DNA, and bending and looping DNA to reach close to the promoter to interact with KorA/ TrbA (whose binding sites are overlapping the promoter) and RNAP, and that it has to be dimeric to perform all these functions from distal binding sites. This also suggests that dimerisation is not necessary for sequence-specific DNA binding, which was also confirmed by DNase I footprinting which showed that KorB-C mutants still had specificity for their DNA target despite their lower affinity (Jagura-Burdzy et al., 1999b). KorB-C mutants show complete protection of the O_B10 on the *trfAp* but only half site protection on *kfrAp* (Kostelidou et al., unpublished). This suggested that monomeric mutants have specificity for the better half of the operator on the basis of the consensus matches to the sequence.

In short, deletions in KorB domains might lead to the disruption that particular domain and affect protein folding or cause its dislocation on the protein surface so that the mutated proteins bind to DNA in a different manner and do not expose regions responsible for cooperativity to contact a second repressor and RNAP.

KorB gene silencing has been reported officially for the first time in this study. KorB spreading is of considerable interest because of its ability to repress transcription at distances of 1.5 kb or more (Bingle et al., 2005). In comparable experiments with bacteriophage P1 it was shown that ParB can spread up to 10 kb from the *parS* sequence that initiates binding, and transcriptional silencing of genes within the sphere of this spreading was observed

(Rodionov et al., 1999). However, the estimated level of ParB (>7000 dimers per cell) and the copy number of P1 (one per chromosome) mean that the amount of ParB available to spread from the single *parS* site is much greater than for KorB. Our highest estimates of KorB level (at most approximately 1500 dimers per cell) would give about 10–15 dimers per O_B site, based on there being 10 plasmid DNA molecules per cell in exponential phase (Thomas and Smith, 1987). The DNA regions contacted by KorB should be limited if each dimer occupies 20 bp of DNA as in DNase I footprinting (Balzer et al., 1992; Williams et al., 1993) and since the KorB surface is dominantly negatively charged (Khare et al., 2004) it should need to recruit basic proteins—for example, plasmid-encoded IncC (Jagura-Burdzy et al., 1999a; Rosche et al., 2000) or nucleoid-associated proteins (Dame, 2005) to contact more DNA by wrapping. This study shows that KorB can silence an adjacent replicon as other ParB proteins do. Also, gene silencing via KorB is potentiated by KorA and TrbA. This result strengthens the possibility that KorB might be sequestered to those sites where there is cooperative interaction with other proteins, but since the KorA and TrbA binding sites flank the KorB binding sites it is surprising that they do not actually limit the silencing process **(Figure 4.14)**. The conclusion from this data however, is that the interaction does not result in sequestration of KorB to a subset of sites on the plasmid. In order to observe gene silencing, KorB expression has to be induced with 0.5 mM IPTG, which is 10 times higher than the amount required to observe repression (as used in *xylE* assays) and gene silencing when KorB and KorA or TrbA are present. KorB resembles ParB homologue Spo0J of *Bacillus* subtilis, which is capable to of spreading up to several kilobases as demonstrated via chromatin immunoprecipitation (Breier and Grossman, 2007), but unlike KorB this spreading does not result in the silencing of genes as shown by DNA microarray (Brier and Grossman, 2007).

Full length KorB is required to silence genes in the absence of other regulatory proteins. However, at higher protein concentration (i.e. 1 mM IPTG) KorB NΔ30 and Δ255 -285 aa can

silence genes on their own, and the same effect is observed at lower protein concentration (i.e. 0.1 mM IPTG) in the presence of KorA and TrbA. Conversely, neither the presence of KorA or TrbA nor the higher concentration of IPTG made any difference in the gene silencing abilities of the rest of KorB deletion mutants. The fact that TrbA/KorA can potentiate P-repression but neither D-repression nor gene silencing for some of the KorB mutants (i.e. C-terminal mutants) shows that such mutants are defective in spreading, bending and looping the DNA. It could be that these mutations make changes in the overall structure of KorB, which result in different conformation of complexes upon binding to DNA and would also be defective in spreading along DNA. This detailed analysis of the domains of KorB opens the door for substitution mutagenesis to find single aa responsible for various functions.

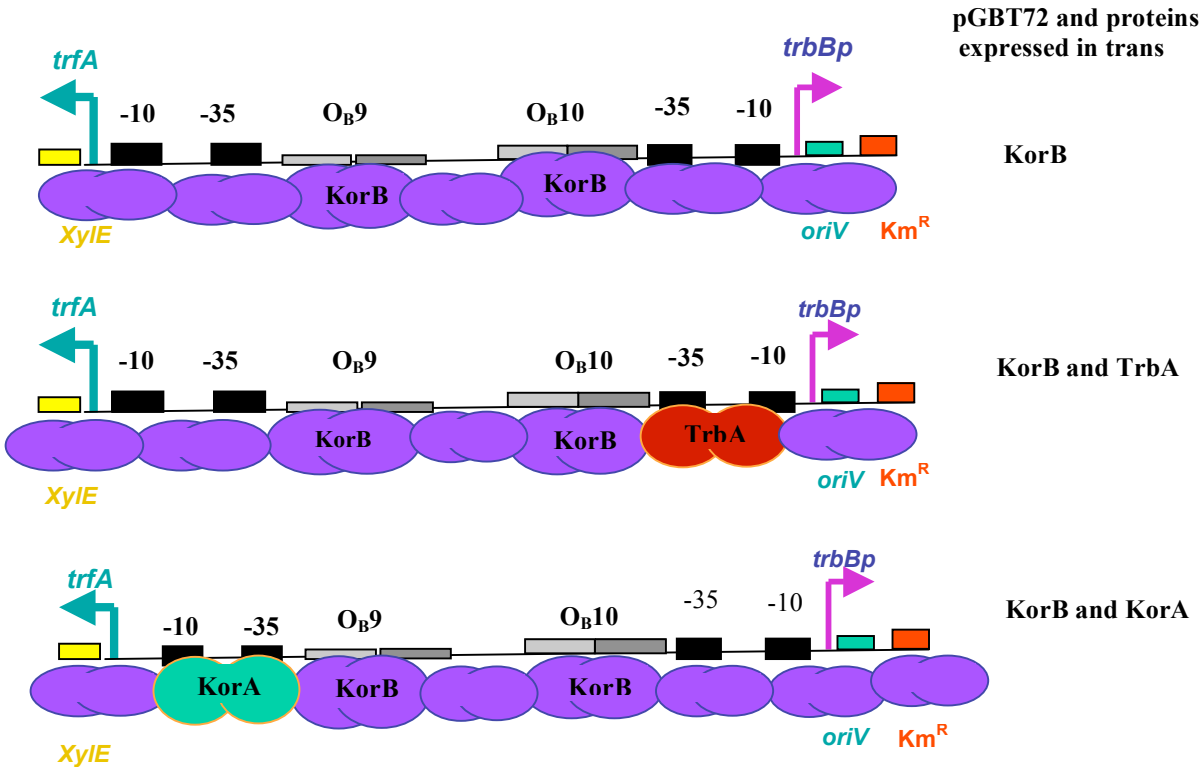


Figure 4.14: Model summarising KorB gene silencing activity. KorB binds and spreads along DNA and can interfere with the expression of genes, such as pSC101 replicon and Km^R. TrbA and KorA potentiate the silencing activity of KorB. In the presence of KorA or TrbA, gene silencing is achieved at lower KorB concentration. This shows that KorA and TrbA do not act as roadblock in gene silencing by KorB.

Chapter 5: KorB amino acids critical for interaction with DNA and other repressor proteins in RK2

5.1 Introduction

Broad host range IncP1 plasmids have complex regulatory circuits that are controlled tightly by heterologous cooperativity between the regulatory proteins. Eukaryotic transcriptional regulation also involves cooperative interaction of proteins in both activation (Merika and Thanos, 2001) and repression (Valentine et al., 1998). Examples of heterologous cooperativity in prokaryotic regulation systems include CRP, MelR (Wade et al., 2001) and CytR (Shin et al., 2001; Pedersen et al., 1991; Chahla et al., 2003) in *E. coli*, PutR in *Vibrio vulnificus* (Lee and Choi, 2006). Cooperative interactions leading to activation or repression involve either direct protein-protein interactions, or modulation of local DNA conformations. Global transcriptional regulation in RK2 (also known as RP4) involves KorA, KorB, KorC and TrbA. KorB shows cooperativity pairwise with KorA or TrbA resulting in an improved repression of the promoters. This cooperativity between KorB and KorA or TrbA controls the functioning of important genes involved in the replication, partitioning and transfer of RK2. The synergy between KorB and KorA is based on the cooperative binding of proteins on DNA (Kostelidou et al., 1999).

KorA N-terminus contains a predicted helix-turn helix domain. Its predicted structure shows similarity to many other bacterial transcriptional regulators. KorA shares a conserved C-terminal domain (CTD) with TrbA repressor (i.e. between position 68 and 96, 29 residues segment with overall 76% similarity and 55% identity), and this domain is also present in the middle of protein KlcB (function unknown) (Jagura-Burdzy and Thomas, 1992; Larsen and Figurski, 1994). The CTD of TrbA and KorA has been shown to be essential for dimerisation

and cooperative interactions with KorB (Kostelidou et al., 1999; Zatyka et al., 2001; Bhattacharyya and Figurski, 2001). The obvious homology of the CTDs of KorA and TrbA **(Figure 5.1)** suggests that this domain may be considered as a cooperativity "module". In this Chapter, the residues of KorB responsible for its interaction with KorA and TrbA are identified. Then the WT and important mutant proteins are analysed further, genetically and biochemically.

Most of the ParB family members can silence genes when their binding site is placed near transcriptionally active genes (Lynch and Wang, 1995; Rodionov et al., 1999; Bartosik et al., 2004; Bingle et al., 2005; Dubarry et al., 2006). This silencing is thought to be a consequence of spreading, which inhibits transcription either by affecting binding of RNA polymerase at the promoter, or by interfering with downstream steps in the initiation of transcription (Williams et al., 1993; Jagura-Burdzy et al., 1999). Some ParB family members are involved in repressing their own and other specific promoters cooperatively with the cognate ParA, and sometimes other proteins. In these cases, the regulation of transcription may involve DNA looping instead of, or in addition to, spreading (Friedman and Austin, 1988; Yates et al., 1999; Kalnin et al., 2000; Bingle et al., 2005).

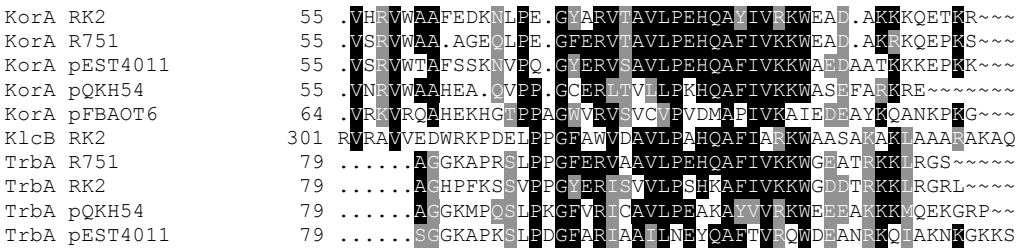


Figure 5.1: Alignment of conserved domains from KorA and TrbA**.** For KorA and TrbA proteins, only one representative sequence from each of the extant IncP-1 subgroups is included to improve clarity, along with the homologue of KorA from the IncU plasmid pFBAOT6 that is not thought to interact cooperatively with its cognate KorB. R751, IncP-1β; RK2, IncP-1α; pEST4011, IncP-1δ; pQKH54, IncP-1γ; pFBAOT6 (IncU).

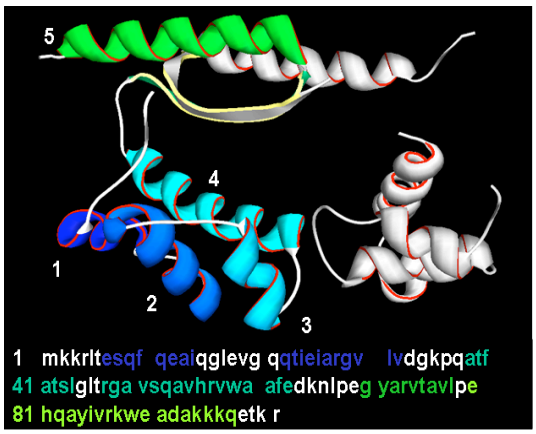


Figure 5.2: Crystal structure of KorA dimer. In the picture only one monomer is coloured for clarity. Residues 65 to 70 are missing in uncoloured monomer. Light blue helices 3 and 4 are the helix turn helix motif. The beta strand and the helix 5 (green) form the dimerisation domain. The structure is asymmetric (White et al., unpublished).

5.2 KorB substitution mutants under study

A KorB internal region was chosen to make substitution mutations because deletion of this region resulted in complete loss of the ability to repress and cooperate with partner repressor proteins (i.e. KorA and TrbA) upon deletion. Furthermore, this region has amino acids which are weakly conserved in other repressor proteins i.e. AsiA (69 – 90 aa) and Rsd A (5 – 26 aa). A set of single substitution, double substitution and deletion mutants in this internal region of *korB* was collected as described in **Chapter 2**; some of these mutants were constructed in previous work, while others were created in this study. The amino acid sequence of that region is presented on **Figure 5.3**, showing the position of the changes. Two acidic residues (glutamic acid - E237 and aspartic acid - D234), two basic residues (arginine – R240 and lysine K244) and two hydrophobic residues (leucines L231 and L245) were chosen as the first targets for site directed mutagenesis because these represented a selection of the "conserved" residues between the three proteins. In the next stage of this work, additional representative of the semi-conserved amino acids were chosen as targets for mutagenesis: glutamic acid – E237,

two leucines – L246 and L250 and phenylalanine – F249. Glycine in position 241 could be important in forming some kind of flexible point, which could be important for conformation of the protein. Therefore we wanted to analyse whether substitution with more bulky amino acids would change activity of KorB.

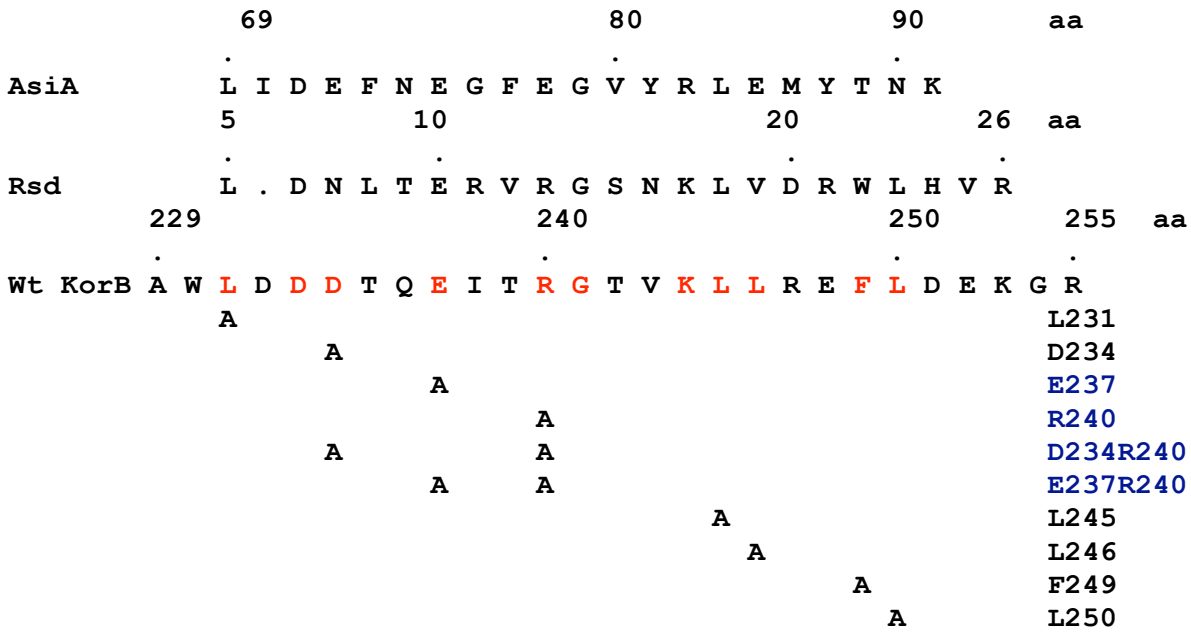


Figure 5.3: The region of KorB between 229 and 254 aa in which substitutions were made. Diagram shows aa sequence of wt KorB, AsiA (aa 66-90) and Rsd (aa 2-26) (coordinates given above sequence). The localization of substitutions in mutants is indicated in red. The position of substitution indicated on right hand side of diagram. The mutants constructed in this study have been shown in dark blue font whereas the ones in black font were constructed previously by Kazimierczak et al., unpublished.

5.3 Results

5.3.1 Circular Dichroism

The secondary structure content of His-tagged KorB (WT/ substitution mutants) was compared by using circular dichroism (CD) spectra at 180 - 300 nm and 25˚C. The path length of the cuvette used was 0.5mm. For each protein type (WT/mutant), a 100 µl sample of 0.5mg protein solution (20 mM Tris, 100 mM NaCl, 10 mM EDTA, pH = 7) was loaded between thin cuvettes, and the spectrum was obtained at 180-300 nm and 25˚C. The data obtained were plotted with Microsoft Excel. Comparison of the spectrum obtained with standard spectra **(Figure 5.4)** indicates that KorB is likely to be largely α-helical, which is consistent with both predicted secondary structure and published crystal structure of the DNA binding domain and C-terminal domain of KorB. Spectra for the KorB mutants (i.e. E237A, E237A K244A, E237AR240A, and E237AD234A) indicate that they retain a spectrum similar to WT KorB as shown in **Figure 5.4**.

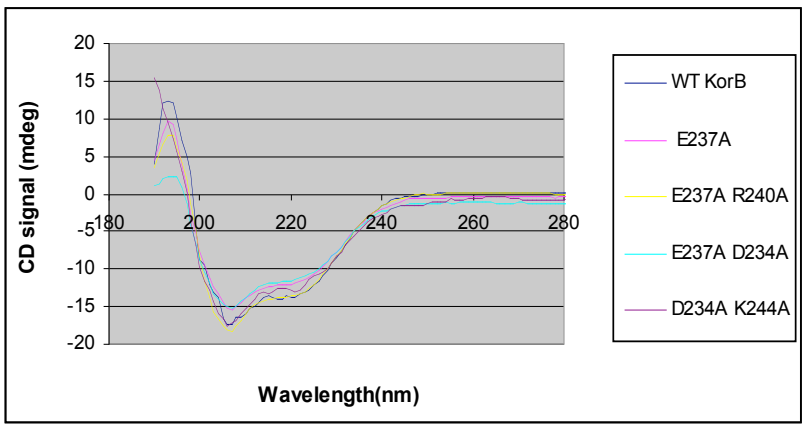


Figure 5.4: Circular dichroism spectrum of KorB (WT/ substitution mutants). This figure shows that there is not big difference between WT and mutant KorB spectrum as all of them are giving negative minima at 210 and 220 nm.

5.3.2 Thermal stability of proteins

The thermal stability of His-tagged KorB (WT, E237A, E237AR240A) was recorded via a circular dichroism (CD) machine, using a temperature range of 20-90˚C. 100 µl samples of 0.5 mg protein solutions (20mM Tris, 100mM NaCl, 10mM EDTA, pH = 7) were loaded between 0.5 mm thick cuvettes, and spectra were obtained at 222nm. The data obtained were plotted with Microsoft Excel. Comparison of WT and mutant KorB indicates that these mutations do not affect the thermostability of KorB.

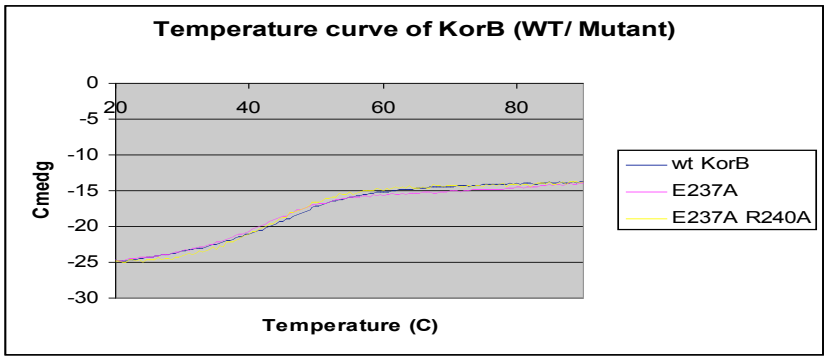


Figure 5.5: Thermal stability curves of KorB (WT/ E237A and E237AR240A). This demonstrates that the substitution mutations didnot make a significant difference in the stability of KorB WT, E237A and E237AR240A

5.3.3 Analytical ultracentrifugation (AUC)

KorB is a dimeric protein as shown previously using gluteraldehyde crosslinking (William et al., 1992). KorB deletion derivatives were tested for their dimerisation ability and oligomeric state in solution using analytical ultracentrifugation (AUC) and data has been recorded in **Table 5.2**. KorB is a dimeric protein with sedimentation value 2.48. This shows that any mutant of KorB with sedimentation value about half of 2.48 would be monomeric. **Table 5.2** shows that all of the KorB substitution mutants i.e. E237A, E237AK244A, E237AD234A, D234AK244A are dimeric.

Table 5.1: Sedimentation values of KorB (WT/mutant)

KorB	Sedimentation value
1. WT	2.48
2. E237A	2.47
3. D234AK244A	2.8

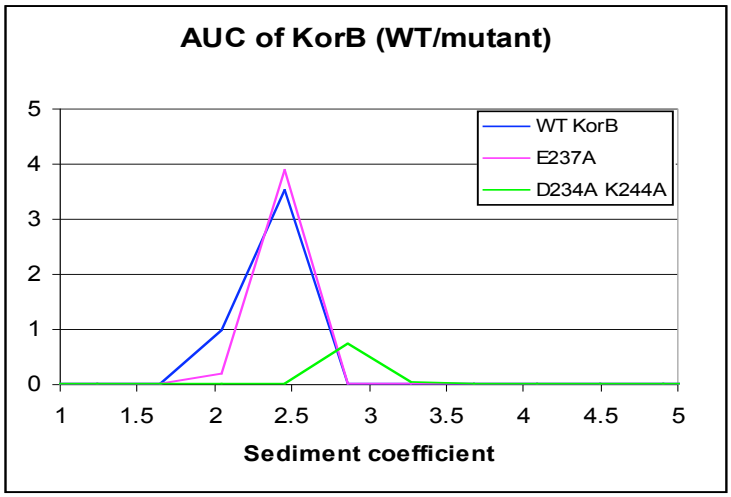


Figure 5.6: Analytical ultracentrifugation of KorB (WT/ substitution mutant). KorB WT and mutants E237A and D234AK244A are dimeric with sedimentation value more than 2.

5.3.4 KorB internal region (235 - 255 aa) modulates its DNA binding

It was observed previously that R240 sits in the major groove near the operator. This led to the suggestion that it is important for operator recognition (Khare et al., 2004). The observation that E237A increases affinity for DNA while K244A decreases it, and that combining the two mutations results in affinity close to WT level, suggests that other charge balancing interactions might exist (Muntaha et al., unpublished). In particular, the loss of DNA binding in the R240A mutant might be due to the negative effects of one of the acid residues nearby (Muntaha et al., unpublished; Khare et al., 2004). We therefore created two additional double mutants, D234AR240A and E237AR240A, and their His-tagged proteins were purified as described in **Chapter 2**.

Electrophoretic mobility shift assays (EMSA) were performed with DNA fragments obtained from pUC18 vector (pKK113) encoding the KorB operator site. *BsaJI* digestion of pKK113 produced three fragments of varying sizes i.e. 1720 bp, 421 bp, 228 bp. Only the 228 bp fragment had an O_B site in it, whereas the other two fragments were controls. The concentrations of KorB WT and mutants were varied.

The results in **Figure 5.7** show that KorB D234AR240A is defective in binding DNA even at higher concentrations. The affinity of KorB E237A and its double mutant E237AR240A for DNA is higher than for KorB WT. It is not consistent with the reduced repression of KorB E237A observed in reporter assays. However, the mobility of the protein-DNA complex was different from that of KorB WT. EMSA experiments showed that the KorB E237A mutation could suppress the defect created by the R240A mutation while D234A could not.

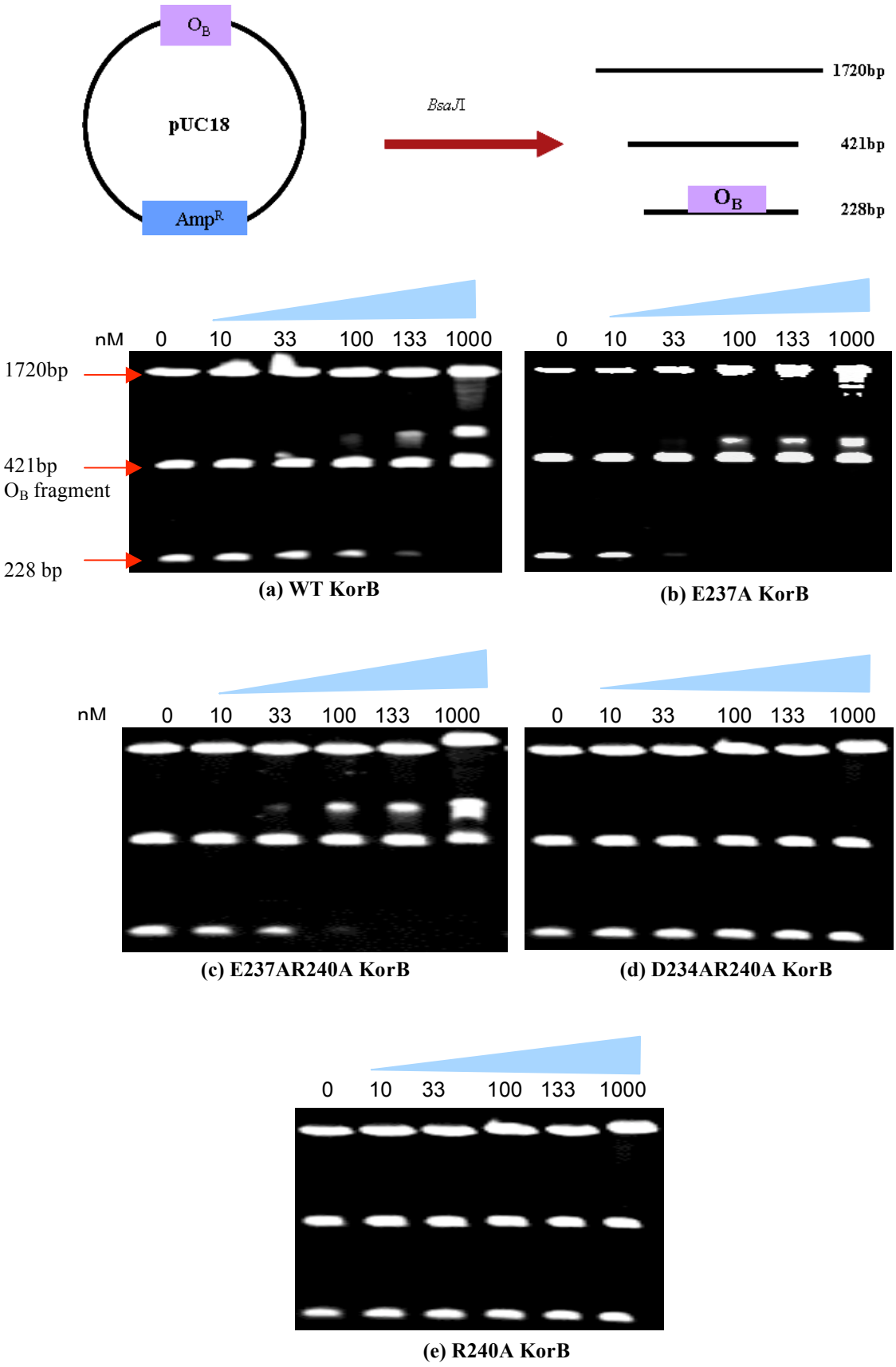


Figure 5.7: EMSAs of KorB (WT/ mutant). pUC18 carrying O_B is digested with *BSaJI* to get three fragments of varying sizes (i.e.1720 bp, 421 bp and 228 bp). Only the 228 bp fragment has KorB binding operator O_B. Other fragments are controls. KorB E237A has higher binding affinity towards DNA than WT. KorB E237AR240A also binds DNA with higher affinity than WT, but slightly lower than E237A. KorB D234AR240A does not bind DNA even at higher protein concentration.

5.3.5 KorB repression and cooperativity at *korAp*

The *korAp* is an important promoter of RK2, as it expresses most of the genes of the central control region. It is classified as a class I promoter where O_B lies approximately 40 bp upstream. KorB (O_B) and KorA (O_A) binding sites lie close to each other at this promoter, and strong cooperativity between them has already been reported. KorB substitution mutants (L231A, E237A, L245A, F249A, L250A and L246A) were chosen to test their cooperativity at *korAp* when O_B is proximal (pDM3.1) or distal (pLB125) to the promoter (Bingle et al., 2005). These reporter plasmid (i.e. pDM3.1 and pLB125) constructs have been discussed in detail in **Chapter 4.** Catechol 2, 3-oxygenase assays were performed using a three vector system in *E. coli* (C600) cells as have been described in depth in **Chapter 4**. All KorB mutants were tested in catechol 2,3-oxygenase assays in order to check their repression and cooperativity activities. First, *E. coli* C600 cells were transformed simultaneously with two plasmids: one of these plasmids was reporter plasmid pDM3.1 (proximal O_B - *korAp* linked to the promotorless *xylE* cassette), and the other was either expression vector pDM1.1 (IncQ replicon, *tacp* empty vector), or pDM1.1 plus *korA* (pSTM11). Second, to the *E. coli* C600 (pDM3.1,, pDM1.1/ pSTM11) background was introduced either a third compatible plasmid pGBT30 (which acts as a negative control and is the vector into which the *korB* ORFs were cloned), or plasmids carrying WT or mutated *korB*. Control assays were performed using the three vector strain carrying pDM1.1 (IncQ replicon) plasmid instead of pSTM11 (KorA expression vector), which enabled us to measure the repression activity of each of the mutated KorB proteins. Expression of KorA and KorB from *tacp* was induced with 0.05mM IPTG and assays were performed three times in triplicate as described in Chapter 2. Strains were set up as presented in **Figure 4.9** of **Chapter 4** and the data from these assays are recorded in **Table 5.2 – 5.3**.

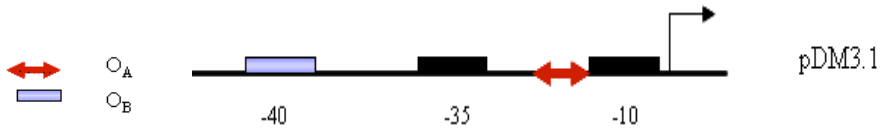
Table 5.2 shows KorB (WT/substitution mutant) repression and cooperativity at *korAp* with proximal O_B. The data presented in the table shows that all of the KorB substitution mutants L231A, E237A, L245A, F249A, L250A repress and cooperate with KorA, except L246A which completely lost the ability to repress (Ri_B value 1.3, Ci value 0.96). KorB L231A and E237A repression was weaker than WT with Ri_B values 200 and 242 respectively, compared to WT Ri_B value 334.

Table 5.3 shows KorB (WT/substitution mutant) repression and cooperativity at modified *korAp* with distal O_B. The data show that all of the KorB substitution mutants lose repression on their own, with Ri_B values less than 5. All of KorB mutants restore repression in the presence of KorA, except F249A (Ri_B=1.1, Ri_{AB}=12, Ci = 1) and L246A (Ri_B=1, Ri_{AB}=8.5, Ci = 0.77), both of which have very low repression and cooperativity index in comparison to WT (Ri_B =27.5, Ri_{AB}=3333, Ci =11).

Table 5.2: *In vivo* activities of *korAp* with proximal O_B in the presence of KorB (WT/ substitution mutants) and KorA.

Reporter Plasmid	**pDM3.1 (*korAp* with O_A and proximal O_B)** [A] *tsp*–O_B centre distance 40 bp				
	XylE activity[X]		Repression index [R]		Cooperativity index[C]
KorB	- KorA	+KorA	KorB Ri_B	KorB + Ri_{AB}	Ci
Empty vector	0.97	0.099	1	9.7	-
WT	0.003	0.00002	334	53900	17
E237A	0.005	0.00003	200	31100	16
L231A	0.004	0.00003	242	3300	14
L245A	0.003	0.00003	319	36100	12
L246A	0.76	0.082	1.3	12	0.96
F249A	0.003	0.00004	340	22600	7
L250A	0.003	0.00001	363	74600	21

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

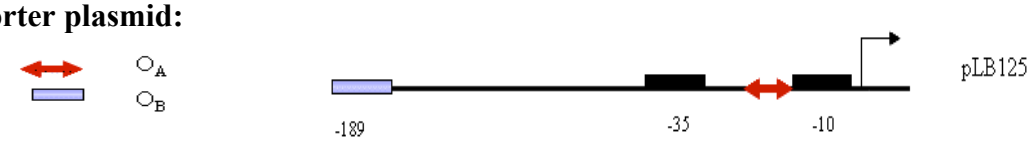
R **Repression index (Ri) =** $\frac{\textit{xylE} \text{ activity - KorA and –KorB}}{\textit{xylE} \text{ activity + KorA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{AB} \text{ (KorA + KorB)}}{Ri_B \text{ (KorB WT/mutant + empty vector)} \times Ri_{AB} \text{ (KorA+ empty vector)}}$

Table 5.3: *In vivo* activities of modified *korAp* with distal O_B in the presence of KorB (WT/ substitution mutants) and KorA.

Reporter Plasmid	**pLB125 (*korAp* with O_A and distal O_B)** [A] *tsp*–O_B centre distance 189 bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	- KorA	+KorA	KorB Ri_B	KorB + KorA Ri_{AB}	Ci
Empty vector	1.1	0.1	1	11	-
WT	0.04	0.0003	28	3333	11
E237A	0.6	0.0008	1.7	1392	72
L231A	0.6	0.008	1.8	1341	67
L245A	0.3	0.0007	4	1641	35
L246A	1	0.13	1	9	0.8
F249A	0.99	0.09	1	12	1
L250A	0.2	0.0006	5	1774	35

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE} \text{ activity - KorA and –KorB}}{\textit{xylE} \text{ activity + KorA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{AB} \text{ (KorA + KorB)}}{Ri_B \text{ (KorB WT/mutant + empty vector)} \times Ri_{AB} \text{ (KorA+ empty vector)}}$

5.3.6 KorB repression and cooperativity at *trbBp*

The *trbB* promoter (*trbBp*) controls the expression of genes involved in the conjugational transfer of RK2. It is a class II promoter, since O_B is placed 189 bp away from the promoter. The distance between O_B (KorB binding site) and O_T (TrbA binding site) is 165 bp.

The same set of KorB mutants (L231A, E237A, L245A, F249A, L250A and L246A) were tested for repression and cooperativity at *trbBp* when O_B is either distal (pMZT39) or proximal (pLB40) to the *trbBp*. Catechol 2,3-oxygenase assays were performed using the three vector system as described in **Chapter 2 and 4** and also in the previous section of this Chapter. Values for *xylE* activity, repression index and cooperativity index are reported in **Tables 5.4** and **5.5**.

Table 5.4 shows that all of the KorB mutants loose repression from distal O_B linked with *trbBp* (reporter plasmid pMZT39) with Ri_B values less than 6. However, repression was restored fully in the presence of TrbA for all mutants except L246A and F249A. KorB L246A and F249 showed complete loss of repression with $Ri_B = 1$, $Ri_{AB} = 1.5$ and $Ci = 0.8$, and $Ri_B = 1$, $Ri_{AB} = 1.4$ and $Ci = 0.8$ respectively.

Table 5.5 shows that all of the KorB mutant except L246A can repress modified *trbBp* with proximal O_B with Ri_B value up to 60 which was increased up to Ri_{AB} value 4090. These results showed once again that KorB L246A is completely defective in repression and cooperativity irrespective of the location of O_B from the promoter. However, KorB F249A can repress on its own when binding to class I O_B. This shows that this residue is important for distal repression, but is not required for cooperativity and proximal repression.

Table 5.4: *In vivo* activities of *trbBp* with distal O_B in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pMZT39 (*trbBp* with O_T and distal O_B)** [A] *tsp*–O_B centre distance 189 bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	KorB	KorB + TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci
Empty vector	1.2	0.8	1	1.5	-
WT	0.02	0.002	60	600	7
E237A	0.7	0.007	3	176	67
L231A	0.4	0.004	3	279	63
L245A	0.5	0.003		387	107
L246A	1	0.8	1.2	1.5	0.8
F249A	1	0.9	1.2	1.4	0.8
L250A	0.2	0.002	5	571	69

A **Reporter plasmid:**

X Values of *xylE* activities from which all these repression indexes have been calculated.

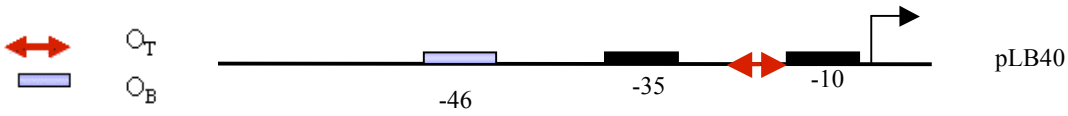
R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

Table 5.5: *In vivo* activities of modified *trbBp* with proximal O_B in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pLB40 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 45 bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	KorB	KorB + TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci
Empty vector	0.9	0.53	1	1.7	-
WT	0.002	0.00003	500	30000	35
E237A	0.03	0.0002	30	4090	80
L231A	0.02	0.0006	38	1470	23
L245A	0.01	0.0006	64	1579	14
L246A	0.99	0.6	0.9	1.5	1
F249A	0.02	0.0007	41	1372	20
L250A	0.01	0.0003	69	2812	24

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

5.3.7 KorB repression and cooperativity at modified *trbBp* with O_B at a distance of more than 1 kb away

As shown previously, some KorB mutants (i.e. E237A L231A, L245A and L250A) are able to repress at class II O_B (when it is 189 bp away from the promoter) only in the presence of KorA or TrbA. These mutants are apparently defective in spreading, providing the opportunity to test the proposal that KorB can act at a distance both by spreading and looping. These mutants were tested for their ability to repress and cooperate with KorA / TrbA when the O_B site is moved as far away as 1.5 kb away from the promoter. KorB was expressed *in trans* to the derivative *trbBp*, with O_B placed at greater distances (up to 1.5 kb) away from the promoter. TrbA was also provided *in trans* alongside controls lacking TrbA.

Catechol 2,3-oxygenase assays were performed to measure repression and cooperativity using the three vector system in *E. coli* C600. However, the reporter plasmids now contain *trbBp* with O_B9 cloned at 278 bp or 636 bp or 1563 bp upstream of the transcription start point, instead of 189 bp as used previously in the native *trbBp* (pMZT39). Otherwise the system was created as described previously. The results of these assays are presented in **Table 5.6.**

The results showed that all of the KorB substitution mutants (i.e. E237A L231A, L245A and L250A) could still repress strongly from O_B at a distance of up to 1563 bp from the target promoter. Their combined repression with TrbA was still very significant as shown in **Table 5.6**.

Table 5.6: *In vivo* activities of modified *trbBp* with distal O_B (distance between O_B and *tsp* is more than 200 bp) in the presence of KorB and TrbA. (a) *xylE* activity (b) repression index.

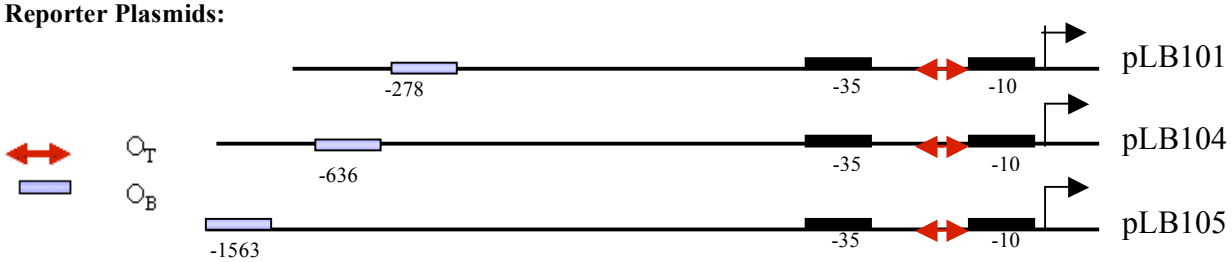
A.

Reporter plasmids (tsp–O_B centre distance)	**pLB101 [A] (278 bp)**		**pLB104 [A] (636 bp)**		**pLB105 [A] (1563 bp)**	
	xylE activity[x]		*xylE* activity[x]		*xylE* activity[x]	
KorB	KorB	KorB + TrbA	KorB	KorB + TrbA	KorB	KorB + TrbA
Empty vector	0.9	0.68	0.89	0.72	1	0.9
WT	0.022	0.0019	0.024	0.0019	0.045	0.0037
E237A	0.69	0.0062	0.69	0.0068	0.69	0.0065
L231A	0.66	0.0055	0.95	0.0089	0.9	0.0079
L245A	0.29	0.0022	0.33	0.0033	0.41	0.0049
L250A	0.26	0.0021	0.32	0.0028	0.36	0.0033

B.

Reporter Plasmids	**pLB101 [A]**			**pLB104 [A]**			**pLB105 [A]**		
korB derivatives *in trans*	**Repression index[R]**		**Ci**	**Repression index[R]**		**Ci**	**Repression index[R]**		**Ci**
	KorB Ri_B	KorB+TrbA Ri_{TB}		KorB Ri_B	KorB+TrbA Ri_{TB}		KorB Ri_B	KorB+TrbA Ri_{TB}	
Empty vect.	-	1.3	-	-	1.2	-	-	1.1	-
WT	41	474	9	37	468	11	22	270	11
E237A	1	145	86	1	130	84	1	154	96
L231A	1	163	92	1	100	89	1	127	104
L245A	3	409	101	3	270	83	2	204	76
L250A	3.5	428	95	3	318	95	3	303	99

A Reporter Plasmids:



X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

5.3.8 KorB E237A can repress proximal promoter even when it binds to the opposite face of the promoter

E237A is apparently defective in spreading. It was tested for its ability to repress from class I O_B facing the opposite face of DNA, by adding 5 bp between O_B and *trbBp*-46. As a control, a *trbBp*-51 construct was used in which another 5 bp were added to bring O_B back on to the same face as the promoter. KorB E237A was expressed *in trans* to WT *trbBp* as well as derivative promoters with O_B placed at -46 and -51 positions from the promoter. TrbA was provided *in trans* alongside controls lacking TrbA. The results in **Table 5.7** and **5.8** show that KorB E237A could still repress strongly and interact with TrbA even if its binding site has been shifted on the other face of DNA.

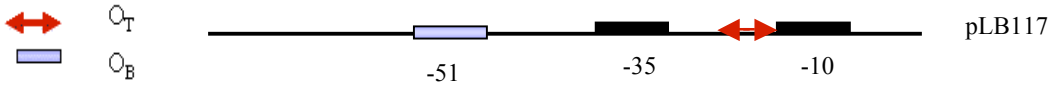
5.3.9 KorB mutants defective in distal repression are also defective in gene silencing

A number of ParB proteins have been shown to silence genetic functions at a distance in a rather non-specific manner and it is suggested that this occurs by spreading along the DNA from a nucleation site. The properties of KorB E237A in particular suggested that it might be defective in spreading. To test this, it was expressed *in trans* to a number of plasmids containing an O_B site and both an antibiotic resistance marker and a replicon that could be targeted by KorB. The controls confirmed that WT KorB can silence genes and that this property is severely curtailed in E237A. Interestingly, when KorA or TrbA were provided in the same cell and when the target plasmid contained a cognate operator, they potentiated the silencing effect – that is, less induction of the *korB* gene was needed to see a silencing effect. Interestingly, this potentiation was observed even when the KorA or TrbA binding site lies between the O_B site and the target for silencing. With KorB E237A the presence of KorA or TrbA partially suppressed the defect in silencing. Results are shown in **Table 5.9**.

Table 5.7: *In vivo* activities of *trbBp* with proximal O_B facing the opposite face of the promoter in the presence of KorB (WT/ E237A mutant) and TrbA.

Reporter Plasmid	**pLB117 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 50 bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	No TrbA	TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci
Empty vector	1.	0.57	-	1.8	-
WT	0.002	0.00003	515	38148	41
E237A	0.026	0.0002	40	4879	68

A **Reporter plasmid:**



X Values of *xylE* activities from which all these repression indexes have been calculated.

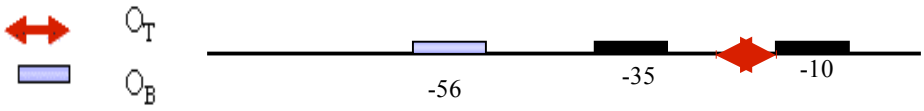
R **Repression index (Ri) =** $\dfrac{\textit{xylE}\text{ activity - TrbA and –KorB}}{\textit{xylE}\text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\dfrac{Ri_{TB}\text{ (TrbA + KorB)}}{Ri_B\text{ (KorB WT/mutant + empty vector)} \times Ri_T\text{ (TrbA + empty vector)}}$

Table 5.8: *In vivo* activities of *trbBp* with proximal O_B facing the same face of the promoter (reporter plasmid constructed by adding 5 bp between O_B and O_T in pLB117) in the presence of KorB (WT/ deletion mutants) and TrbA.

Reporter Plasmid	**pLB118 (*trbBp* with O_T and proximal O_B)** [A] *tsp*–O_B centre distance 55 bp				
	xylE activity[X]		Repression index[R]		Cooperativity index[C]
KorB	No TrbA	TrbA	KorB Ri_B	KorB + TrbA Ri_{TB}	Ci
Empty vector	0.96	0.65	-	1.5	-
WT	0.002	0.00003	436	38400	59
E237A	0.04	0.003	22	310	10

A **Reporter plasmid:**



pLB118

X Values of *xylE* activities from which all these repression indexes have been calculated.

R **Repression index (Ri) =** $\frac{\textit{xylE} \text{ activity - TrbA and –KorB}}{\textit{xylE} \text{ activity + TrbA and/or + KorB (WT/mutant)}}$

C **Cooperativity Index (Ci) =** $\frac{Ri_{TB}\ (\text{TrbA + KorB})}{Ri_B\ (\text{KorB WT/mutant + empty vector}) \times Ri_T\ (\text{TrbA + empty vector})}$

Table 5.9: Gene silencing by KorB (WT/mutant)

Resident plasmids used for gene silencing	**pGBT72**			**pGBT73**		
	Regions inserted in pSC101-based plasmids (pGBT72 and pGBT73) and concentration of IPTG needed to induce silencing[b]					
Repressors present *in trans*[a]	**O_B *trfAp* O_A >** / **< O_T *trbBp* O_B**			**O_B *trbBp* O_T >** / **<O_A *trfAp* O_B**		
KorB (WT/mutant)	Empty Vector	KorA	TrbA	Empty Vector	KorA	TrbA
1. Empty Vector	-	-	-	-	-	-
2. WT	+++	++	++	+++	++	++
3. E237A	-	-	-	-	-	-

\- = No silencing
\+ = Gene silencing
+++ = Gene silencing at 0.5 mM IPTG
++ = Gene silencing at 0.05 mM IPTG

a. Repressor genes under the control of the tac promoter were provided from the following plasmids: pMMV811 (KorB), pSTM11 (KorA), pLB25 (TrbA)
b. Gene silencing was assessed as described in Methods.

5.3.10 DNA binding is not sufficient for KorB to repress the promoters

KorB D234AK244A binds DNA stronger than WT (DNA binding reaction was set up same as described in chapter 2). Previously, it was found to be defective in repression at distal and proximal O_Bs at *korAp* and *trbBp*. A new synthetic *trfAp-1* was constructed **(Figure 5.9)** by annealing two oligos and ligating them into *BamH*I digested pPTO1 vector to link promoter-less *xylE* cassette. KorB D234AK244A didn't show any repression even when O_B was overlapping the promoter as shown in **Table 5.10**.

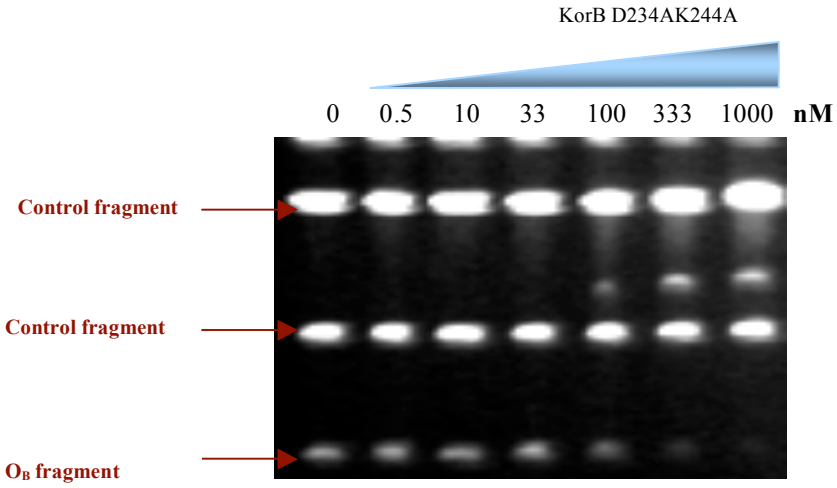


Figure 5.8: EMSAs showing KorB D234AK244A binding specifically with DNA having O_B.

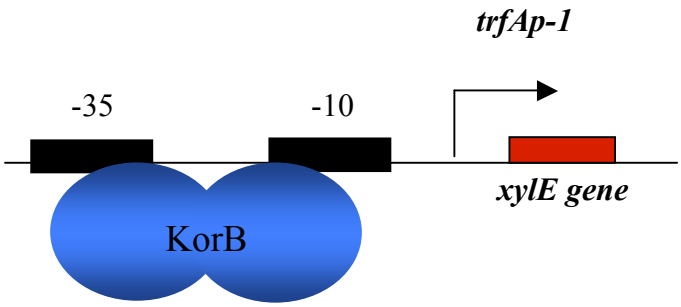


Figure 5.9: Genetic map of pSTM2 used in reporter gene assays. KorB binding site is overlapping the synthetic *trfAp-1* from which *xylE* gene is being expressed.

Table 5.10: *In vivo* activities of synthetic promoter *trfAp*-1

Reporter plasmid	**pSTM2 (O_B is overlapping the -10 region of *trfAp-1*)**	
	xylE activity[1]	Repression index
	IPTG (mM) 0.05 0.1	IPTG(mM) 0.05 0.1
1. Empty vector	0.97 0.099	1.21 0.15
2. KorB WT	0.297 0.00019	0.25 0.00066
3. KorB D234AK244A	0.48 0.00031	0.83 0.29

5.4 Discussion

This chapter reports a number of important findings regarding the proximal and distal repression mechanisms by which the IncP-1 ParB family protein KorB regulates the expression of genes. This study confirms the observation of Lukaszewicz et al., (2002) that KorB binding to the upstream of the -35 region of a promoter is not sufficient for transcriptional repression, since combined point mutations D234A and K244A in KorB result in the complete loss of repression but retain the normal DNA binding activity. These results are in line with the previous conclusions that KorB must actively interfere with RNAP activity once it has bound to the DNA at the *korAp*, and tend to contradict the observation that KorB excludes RNAP from the *trfAp* unless somehow KorB can bind to similarly positioned O_B sites in different ways so as to exclude RNAP in some contexts but not others.

This study also shows that the region around R240 is not necessarily involved in operator recognition as proposed by Khare et al, (2004), since the defect in binding of the R240A mutation can be suppressed by the E237A mutation. This suggests that the role of R240 is to help attract KorB to the DNA and to counteract the repulsion between the backbone and the E237 side chain. The proposal that R240 was essential for operator recognition was not consistent with the normal principles of bacterial gene regulation and it seems sensible at this stage at least to revise the conclusion. Nevertheless, the observations are not entirely negative because they suggest an alternative role for R240 that appears to be critical in allowing E237 to perform its function. From the available crystal structure it appears that E237A sits close to the phosphate backbone and between the subunits of KorB that bind to the two halves of an operator. It is known that KorB binding to DNA causes bending, and we propose therefore that this can be assisted by the basic residues that help to grip the DNA on either side of the

acid residues that tend to repel the DNA, thus acting as an electrostatic lever. On its own this protein has lost the ability to act at a distance – it does not repress *trbBp* through O_B9 or *trbAp* and does not cause silencing. This suggests that the protein is defective in spreading on the DNA away from an O_B site. The ability of KorA and TrbA to stimulate silencing even in the direction that might be blocked by the second protein – implies that KorB can spread past a "road-block".

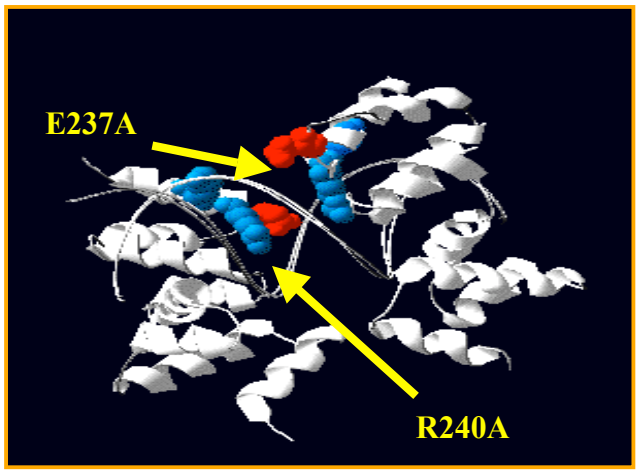


Figure 5.10: Views of KorB DNA binding domain-O_B (KorB-O) complex structure (adopted from Khare, *et al*., 2004). Location of E237 is shown at the interface between KorB monomers. KorB-O clamps the operator and E237 creates electrostatic stress.

This chapter also reports repression of a number of other KorB substitution mutants (i.e. L231A, L245A, L246A, F249A and L250A) in the internal region of KorB, which was suggested (along with experimental proof) to be important for repression as well as cooperativity with KorA and TrbA in Chapter 4. All mutated *korB* ORFs were cloned first into pGEMT-E and then into pGBT30 and pET vector. All of the KorB substitution mutants were able to bind DNA and their dimerisation and oligomerisation and thermal stability did not vary much from WT KorB. This implies that there are no major conformational changes

and that all analysed proteins were in dimeric form. However, the data do not exclude the possibility of minor changes in conformation that may not be detected in these tests.

Repressor activities of KorB substitution mutants were analysed at *korAp* and *trbBp* with O_B located at different positions. At *korAp,* class I O_B was localised at 40 bp upstream of the *tsp* and at modified *korAp* O_B was localised at a 189 bp distance. At *trbBp* class I O_B was placed at -41, -46 and -51 bp positions and class II O_B was localised at -189, -278, -636, 1563 bp upstream of the promoter. Single non-polar, neutral KorB residue, leucine (L246) was completely affected for repression and cooperativity with KorA and TrbA at both class I and class II O_Bs. This mutant binds DNA with higher affinity than WT as determined previously (Kazimierczak et al., unpublished). KorB F249A lost the ability to repress and cooperate at class II O_B, but it repressed and cooperated fully at class I O_B. KorB mutants defective in distal repression from class I O_B (i.e. E237A, L231, L245A, L250A) showed strong repression on their own at class II O_B. However their repression was increased in the presence of KorA/TrbA at both class I and class II O_Bs, which could be due to the improved interaction with RNAP. This also shows that cooperativity is not dependent on the ability to repress.

One possible explanation of the cooperativity defective KorB substitution mutants could be that these mutations lead to the disruption of this region or cause its delocalisation on the protein surface, resulting in the loss of cooperativity. However some mutations do not have any negative effect on cooperativity, which suggests that the internal region might not be directly involved in interaction with KorA and TrbA. It is possible that mutated proteins bind DNA in a different manner and do not expose the regions required to contact the second repressor protein. In other words, these substitutions may lead to local changes in protein

structure which affect protein folding, resulting in the displacement of the region involved in cooperative interaction from the position required for active interaction between two proteins.

There are several possible explanations for the observed differences in repression at class I and class II promoters. Repression at a distance may require KorB properties which are different from those needed for repression at class I promoters. This may include diverse mechanisms of repression at class I and class II promoters, or requirement for different protein regions. Previous *in vitro* studies have shown that at *trfAp* binding of KorB blocks RNAP from binding to the promoter (Jagura-Burdzy and Thomas, 1997). However, once RNAP was bound to the promoter, KorB cannot replace it. On the other hand, at *korAp* it seems clear that KorB represses by blocking isomerisation of RNAP-promoter complexes (Williams et al., 1993). The mechanism of repression at class II promoters is not known. It was proposed that KorB needs to contact two binding sites simultaneously or has to be able to interact with sequences flanking the operator and cause distortion of DNA (Jagura-Burdzy et al., 1999b). Footprinting analysis at O_B9 located upstream of *trbBp* suggested that KorB might wrap DNA around itself, and may possess more than one region that interacts specifically with DNA (Jagura-Burdzy et al., 1999b). Perhaps this additional DNA recognising region within KorB is required for repression at a distance but not for proximal repression, which could be 255-285 aa based on the analysis in **Chapter 4**. KorB E237A DNA footprint analysis showed that it wraps DNA around at higher concentration in a different manner than WT (Kazimierczak et al., unpublished). The distortion of DNA by KorB may be dependent on its ability to oligomerise, and the internal substitution may affect this ability, which can also be explained by an inability of KorB E237A to silence neighbouring genes.

KorA is a better repressor than KorB on its own. KorA binding site O_A is located in the promoter region, whereas KorB binding site O_B is located either up or downstream from the promoter. In order to test if KorB achieves better repression when its binding operator O_B is overlapping the promoter (as is the case with KorA binding operator O_A), an artificial promoter (*trfAp*-1) construct was made where O_B was placed overlapping the -10 region of the promoter, and repression was measured. Results showed no effect on the level of KorB repression. This suggests that the KorB region required for interaction with RNAP is not located in the right place. KorB D234AK244A binds DNA with higher affinity than WT, but shows no repression even when O_B is overlapping the promoter. This implies that DNA binding is not sufficient for repression.

Table 5.11: Summary of KorB mutants and their functions.

KorB	Conclusions
E237A	• Defective in silencing can not repress at a distance when alone but can in presence of TrbA – strong evidence of looping. • The fact that KorA and TrbA don't potentiate gene silencing by E237A but do potentiate its repression indicates strongly that gene silencing is because of spreading instead of looping. • Balance of charge in the region from 230 to 250 aa modulates DNA binding, repression and gene silencing activity. • In order to repress KorB needs to do more than just binding DNA.
L246	• required for repression.
F249	• required for repression and cooperativity at a distance.
E237A **L231A** **L245A** **L250A**	• KorB mutants which were weak repressors at class II O_B but repress strongly at class I and show cooperativity at both class I and II O_B

Chapter 6: KorA interaction with KorB *in vitro*

6.1 Introduction

KorB is a 358 amino acids, negatively charged (-21) protein. It forms dimers in solution but higher multimers have also been observed. It belongs to the ParB family of DNA binding proteins, which are involved in the partitioning of bacterial chromosomes and plasmids. It negatively regulates transcription from adjacent promoters. KorB plays a dual role as a regulator of many RK2 circuits and a part of the partitioning apparatus. It specifically recognizes and binds to 12 operators. KorB operators (O_B) do not occur only in promoter regions, but also in intra- and intergenic regions. The sites found in promoter regions are either 4-5 bp upstream of the −35 hexamer (for *trfAp*, *korAp* and *klaAp*, Class I O_B) or further upstream or downstream of promoters, but within 80-180 bp of the tsp (for *trbBp*, *kfrAp* and *kleAp*, class II O_B). KorB can repress the promoters where an O_B is found but its role in the other regions has not been identified. KorB binding sites are well conserved on the relatives of RK2, not only in plasmid R751, the archetype of the $IncP_\beta$ subgroup, but also in all other IncP-1 subgroups identified to date; and they thus must play an important role in plasmid regulation and control (Thorsted et al., 1998; Kostelidou et al., 1999; Bingle et al., 2005).

KorB cooperates with other global regulators (such as KorA and TrbA) in regulation of genes involved in the stable inheritance of plasmid by controlling replication and plasmid partitioning functions. KorA and KorB are encoded in the central control region (*ccr*) (Bechhofer et al., 1983, Kornacki et al 1987 and Theophilus et al., 1987). KorA (101 aa) is a protein with an estimated molecular mass of 11,000 Da and an overall positive charge of +4 (Pansegrau et al., 1994). It is dimer in solution but higher multimers have also been observed. KorA specifically recognizes and binds to 7 operators (consensus sequence: 5′-

GTTTAGCTAAAC-3) in the *par* region. Class I (higher affinity) KorA operators occur at *trfAp*, *korAp* and *klaAp*, while class II O_A sites (lower KorA affinity due to sequence degeneracy) are found at *kleAp*, *kleCp*, *klcAp* and *kfrAp* (Jagura-Burdzy et al., 1995). It plays an important role as a regulator in the replication and inheritance of RK2 and interacts with KorB. Five of the KorA operators lie near operators of KorB at *korAp, trfAp, kleAp, klaAp, and kfrA* . The presence of both KorA and KorB leads to severely decreased promoter activity (Kostelidou et al., 1999). The C-terminus of KorA interacts with KorB. At *korAp,* 3 to 4 fold cooperativity has been recorded between KorA and KorB (Kostelidou et al., 1999). The structure of KorA has been recently solved by White et al., unpublished **(Figure 6.1)**.

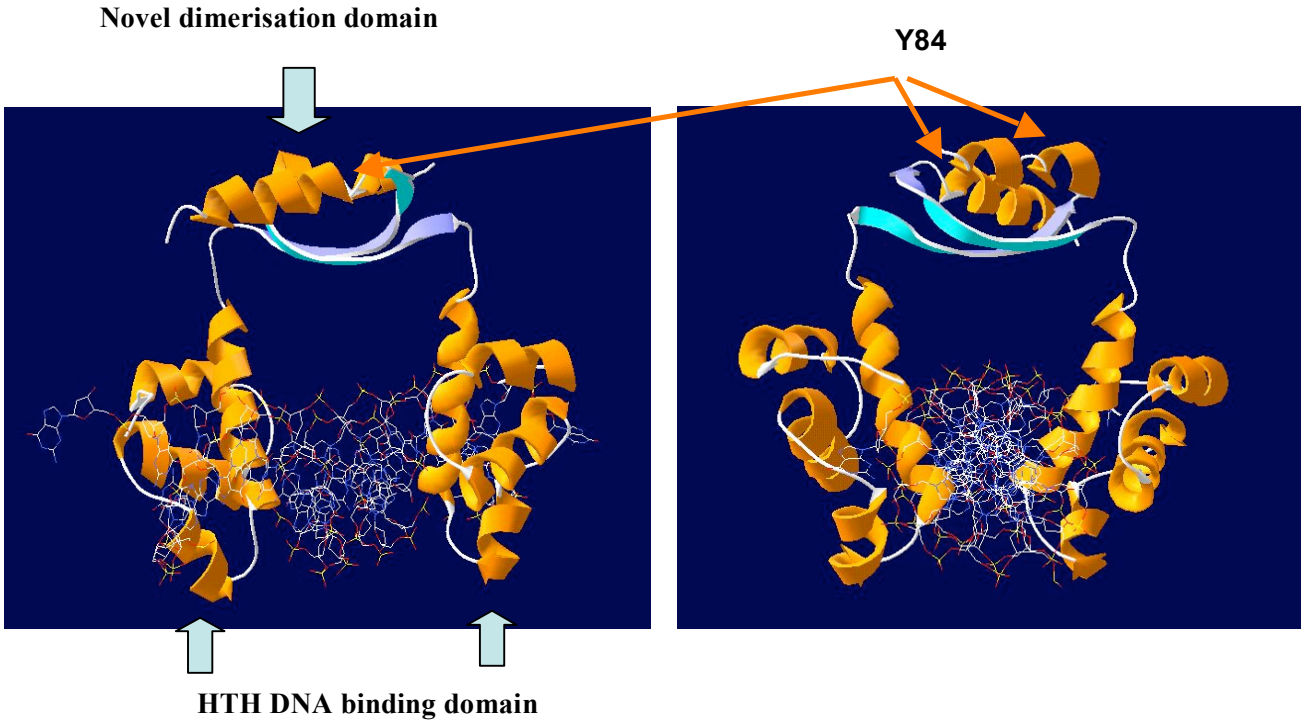


Figure 6.1: KorA-DNA crystal structure. The structure is asymmetric. Side and end view of KorA bound to DNA and showing location of Y84 (White et al., unpublished)

6.2 Results

6.2.1 Protein purification

His-tagged KorA WT and Y84A were purified as described in **Chapter 2**. Both of the proteins produced were clean and in good amount. These proteins were very soluble and were used to study interaction with KorB etc.

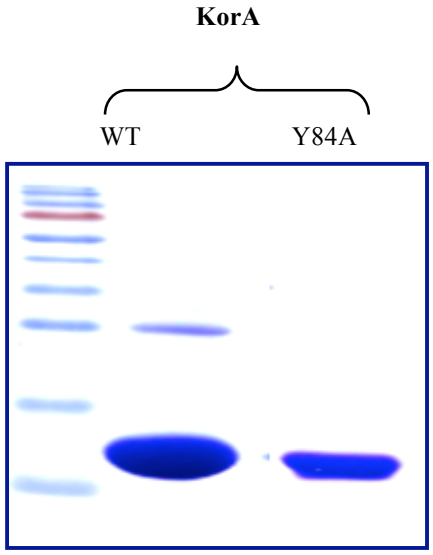


Figure 6.2: Purification of His-tagged KorA (WT/mutant) by using Ni-agarose column.

6.2.2 Circular Dichroism

The secondary structure content of His-tagged KorA (WT and Y84A) was determined by using circular dichroism (CD) spectra at 180-300 nm and 25˚C. The path length of the cuvette was 0.5mm. About 100 µl samples of 0.5 mg protein solution (20 mM Tris, 100 mM NaCl, 10 mM EDTA, pH = 7) were loaded between thin cuvettes and the spectrum was obtained. The data were plotted to make graphs using Microsoft Excel. Comparison of the spectrum with the standard spectrum measured for proteins with essentially 100% α helical, β sheet or random

structure indicate that KorA is likely to be largely α helical which is consistent with both predicted secondary structure and the unpublished crystal structure of KorA (White et al., unpublished) shown in **Figure 6.2**.

The CD spectrum in **Figure 6.3** also showed that the secondary structure of KorA Y84A does not differ from the WT and it is also largely α helical.

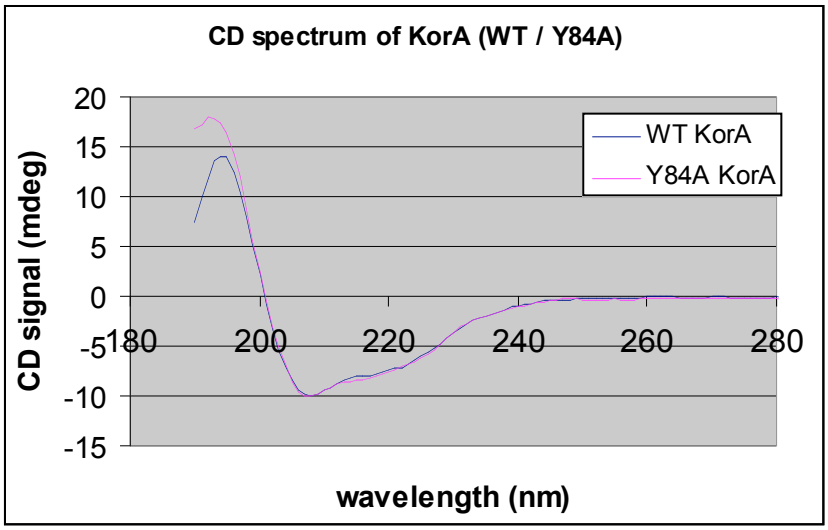


Figure 6.3: CD Spectrum of KorA (WT/mutant). KorA Y84A does not vary much from WT and they are in good order of structure.

6.2.3 Analytical ultracentrifugation (AUC)

KorA is a dimeric protein as shown previously using gluteraldehyde crosslinking. KorA (WT/mutant) oligomeric state was determined in solution using AUC. WT KorA is a dimeric protein in solution and it gave sedimentation value 2.5. **Table 6.1** shows that KorA Y84A is also dimeric with sedimentation coefficient = 2. Any KorA mutant with sedimentation value approximatey half of 2.5 will be monomeric.

Table 6.1: Sedimentation coefficient of KorA (WT/mutant)

KorA	Sedimentation coefficient
WT	2.5
Y84A	2

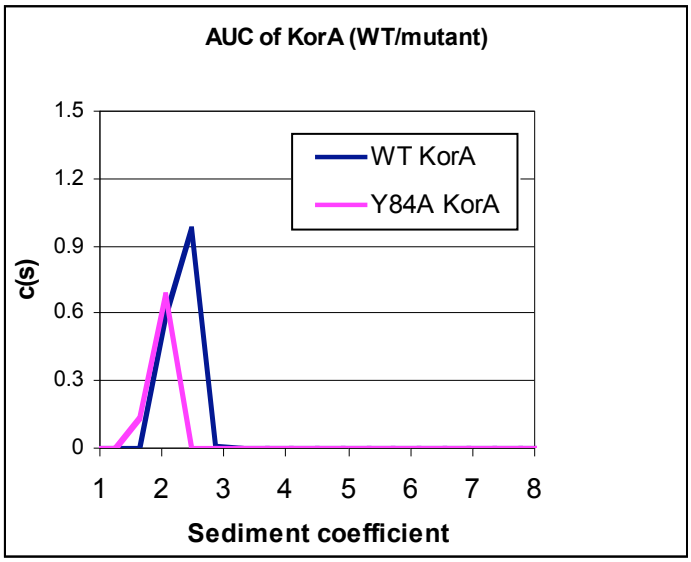


Figure 6.4: Analytical ultracentrifugation of KorA (WT/ substitution mutant). KorA WT and mutants Y84A are dimeric with sedimentation value 2 or more.

6.2.4 KorAY84A is defective in cooperativity with KorB

It has been previously demonstrated in our lab that KorB binds preferentially to DNA with KorA bound *in vitro* (Kostelidou et al., 1999). Further studies by Bingle et al., 2008 showed via *in vivo* studies using *xylE* assays that KorA Y84 aromatic residue is critical for the interaction of KorA with KorB To check whether the Y84A mutation affects this interaction with KorB in an electrophoretic mobility shift assay, WT and Y84A mutant His-tagged KorA as well as KorB were purified as described in **Chapter 2**. They were tested using *BsaЛ*-cut plasmid pKK331 (described in detail in **Chapter 5**) DNA that releases an approximately 228 bp fragment (having O_B) and two control fragments of 421 bp and 1720 bp. A key difference from our previous assays is that non-radioactively labelled DNA has been used in this work, visualizing the DNA by ethidium bromide fluorescence, so the DNA concentrations used were higher and thus the amount of protein needed for retardation appears to be higher than we have reported previously (Kostelidou et al., 1999). Initially the varied amounts of both KorA and KorB were used separately to identify the concentration at which KorA gave partial retardation and the concentration at which KorB first gave significant retardation. When KorA was present, KorB binding to the 228 bp DNA fragment (having O_B) was reproducibly enhanced by KorA WT, with a super-shift by KorB being observed at 33 nM. By contrast, the presence of KorA Y84A had consistently little effect on KorB binding indicating that the enhancement in KorB binding by WT KorA was not simply due to the presence of a basic protein, but that it was due to a specific interaction that has been affected by the Y84A mutation **(Figure 6.5)**.

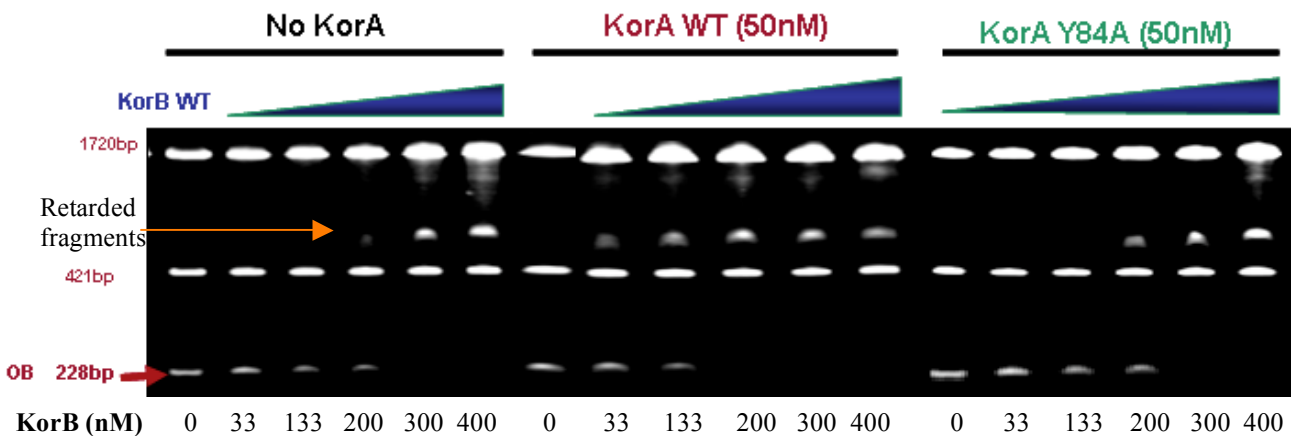


Figure 6.5: KorB binding to 200 bp DNA fragment (having O_B) in the presence or absence of KorA (WT and Y84A). Each protein had previously been titrated to determine suitable concentrations. Proteins were added separately or together as described in material and methods. The difference between the effect of WT and mutant KorA was not always as clear as this, but quantitative analysis of band intensities and pooling of the data showed that it was a significant and reproducible effect.

6.2.5 KorA interaction with KorB in the absence of DNA

Since the NMR data indicate that KorA and KorB undergo direct contact I performed pull-down assays to determine whether the interactions were strong enough to result in co-purification of the two proteins. Crude lysate of bacteria expressing His-tagged KorB was mixed with crude lysate from cells expressing WT or mutant KorA. The experiment was performed this way round because even non-His-tagged KorB can be retained on Ni-Agarose whereas KorA does not bind unless His-tagged. KorA WT was found to copurify with His-tagged KorB, thereby confirming a direct protein-protein interaction.

Conditions were optimised to have the same amount of WT and mutant of KorB proteins. NΔ200, NΔ250 KorB express at low level compared to WT KorB and thus double of the

amount of culture were used for these proteins so that the amount of KorA pulled down would depend only on its interaction with his-KorB rather than its amount.

Some proteins have affinity for binding Ni-agarose. In order to do this experiment and to see if non His-tagged proteins can be pulled down by His-tagged proteins, non His-tagged protein shouldn't have affinity for Ni-agarose. First of all KorB was expressed as non-his tagged and it was found that it was binding to Ni-agarose. Non-his tagged KorA does not bind Ni-agarose. This is why it was decided to express KorB (WT/mutants) as His-tagged and KorA as non His-tagged protein.

Deletion derivatives of KorB were used for pulled down assays to see which mutants of KorB can still pull down KorA. KorB was His-tagged whereas KorA was non His-tagged. His-tagged KorB proteins (WT/ N-terminal mutants) pulled down by Ni-agarose were run on SDS PAGE to see if any other protein had been co-purified. Western blot of SDS PAGE was performed using anti KorA to detect the KorA band.

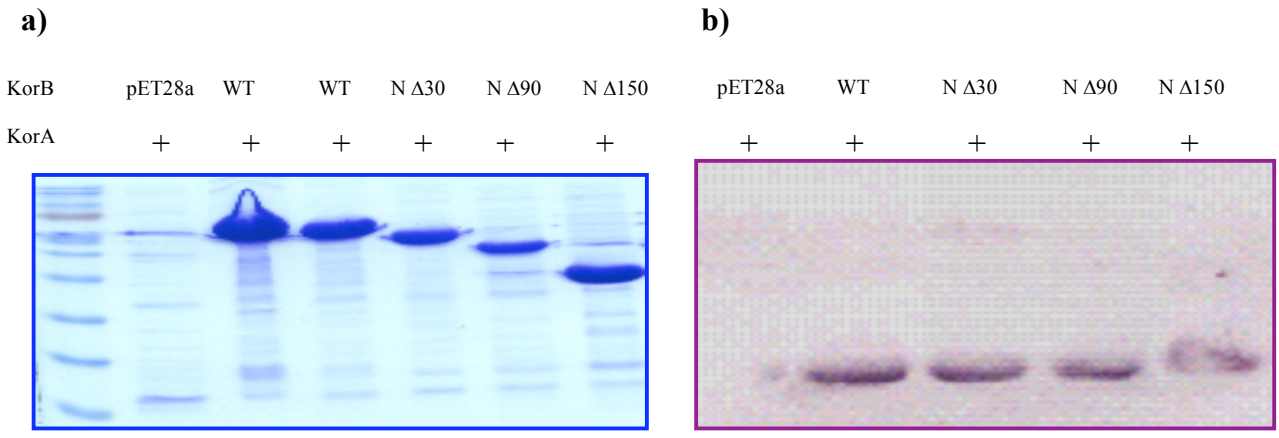


Figure 6.6: KorA pulldown by His-tagged KorB (WT/ N-terminal mutants) **(a)** SDS PAGE of pulled down proteins **(b)** Western blot of acrylamide gel using anti KorA.

It was found that His-tagged-N-terminal derivatives of KorB pulled down non His-tagged KorA as strongly as His-tagged WT KorB. EMSA and NMR analysis have reported same results that the removal of amino acids up to 150 aa from the N-terminus of KorB does not affect its ability to interact with KorA.

In order to narrow down the region of KorB that interacts with KorA NΔ200, NΔ250, CΔ60, CΔ87, Δ225-255, DNA binding domain of KorB proteins were used to pull down non-his KorA . It was found that monomeric CΔ60, CΔ87 KorB can pulled down KorA as strongly as WT KorB whereas NΔ200, NΔ250, Δ225-255 and DNA binding domain of KorB were weak in interacting and thus pulling down KorA.

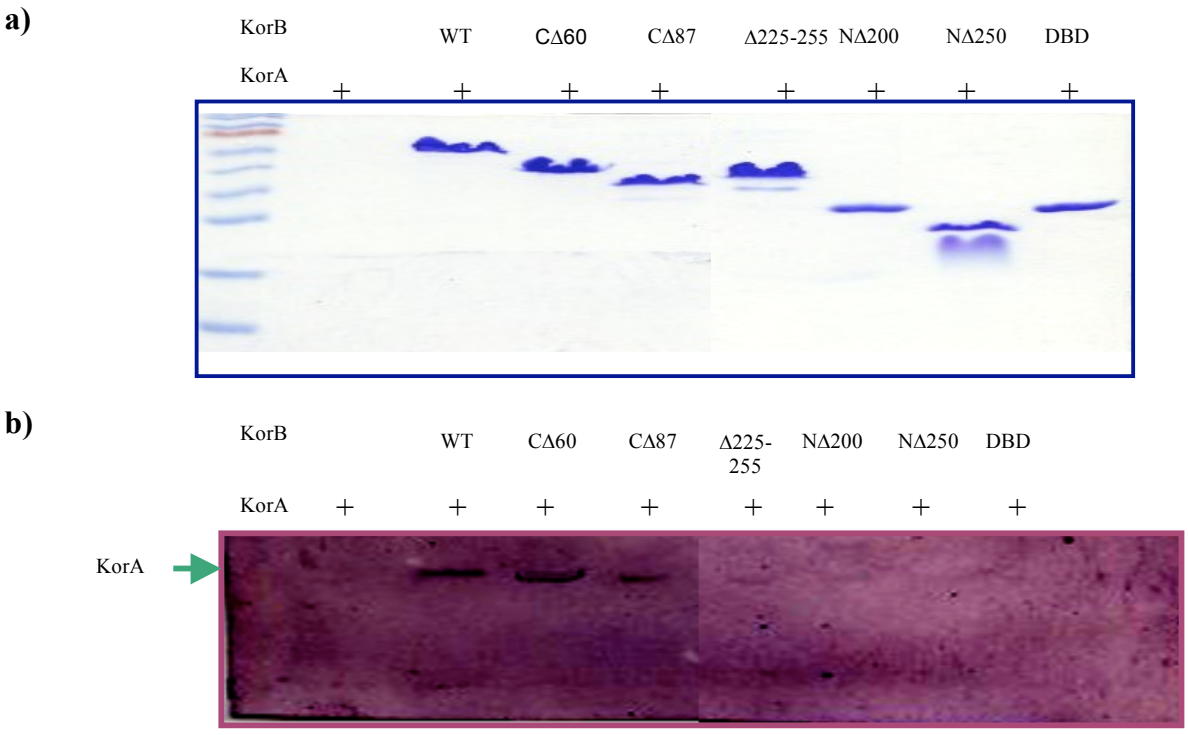


Figure 6.7: KorA pulldown by His-tagged KorB (WT/ mutants) (a) SDS PAGE of pulled down proteins (b) Western blot of acrylamide gel using anti KorA.

Table 6.2: Strength of KorA binding to KorB (WT/mutant) in KorA-KorB complex in the absence of DNA as measured by protein co-purification assays.

KorB	**Pulled down KorA band strength**[a]
No KorB	-
WT	+++
N Δ30	+++
N Δ90	+++
N Δ150	+++
N Δ200	+
N Δ250	+
C Δ60	+++
C Δ87	+++
DBD	+
Δ225-285	+

a. Pulled down KorA band strength as observed from western blot

- = No KorA binding to KorB
+ = very weak KorA binding to KorB
++ = weak KorA binding to KorB
+++ = strong KorA binding to KorB

Based on the results presented in **Figure 6.6 - 6.7** and **Table 6.2**, it is obvious that the region of KorB that interacts with KorA lies somewhere between 150 to 271 aa of KorB.

6.3 Discussion

The results presented in this Chapter demonstrate that there is a direct and specific interaction between KorB and KorA *in vitro* whereas interaction *in vivo* was done by Bingle et al 2008. This interaction has also been detected in purified solutions of KorA and KorB using co-purification assays **(Figure 6.6 and 6.7)**. This is in line with previous predictions based on the conservation of this region between the two repressor proteins, KorA and TrbA, that show cooperativity with KorB and deletion studies on these proteins that implicated the CTD as important for this (Kostelidou et al., 1999; Zatyka et al., 2001). Mutational analysis of the region of KorA involved in this interaction identified one residue, tyrosine 84, as not essential

for repressor ability of KorA, but essential for the interaction between KorA and KorB (Bingle et al 2008) and helped to connect the *in vitro* interaction between KorA and KorB (seen by pulldown assays and EMSA in **Figures 6.6-6.7**) with the ability of KorA and KorB to cooperate in repression. Work from this lab in the past has also shown that KorA with deletion aa 84 to 101 still retained some cooperativity in DNA binding with KorB *in vitro* (Kostelidou et al., 1999). Consistent with all these observations is the fact that the ability of KorA to potentiate KorB binding to a 228 bp DNA fragment containing operators for KorB was reduced by the Y84A mutation **(Figure 6.5)**. It is likely to be the aromatic ring that is critical for the interaction with KorB. It could be that Y84 protrudes from KorA so that it can contact a suitable part of the surface of KorB. From the titration with increasing KorB concentration it appears that each KorB dimer can contact two KorA dimers and that this contact affects the signal for both Y84 residues in each KorA dimmer. This is consistent with KorB having two sides, each of which can simultaneously contact KorA, possibly contacting both subunits at once or singly but in fast exchange. The existence of such an interaction, if it also occurs when the proteins are bound to DNA, raises questions about the flexibility of this interaction, since the DNA and the orientation of the proteins should add constraints to the interaction. The predicted size of KorA (101 aa; monomer diameter if approximately spherical of 2.5 nm) and the known size of KorB bound to DNA (DNA binding domain, 140-250 aa; monomer as a slightly extended sphere with a diameter of approximately 3 nm; Khare et al., 2004) and DNase I foot-printing of KorA and KorB (Jagura-Burdzy and Thomas, 1995; Williams et al., 1993) when their operators are present with 33 bp between their centres is consistent with them being separated by approximately two turns of the DNA helix. Dimers are therefore likely to be close but not in direct surface-surface contact unless one or both proteins have an extended structure (for example the dimerisation domains) that can reach out to the other protein or can bend the DNA to bring the proteins closer. Since both repression and cooperativity are unaffected by insertion of an additional 5 bp between the operators

which should move the relative positions of these proteins by 180^{o} (Bingle et al., 2005) it seems unlikely that the proteins are relatively rigid shapes that interact simply by KorA- or KorB-induced DNA bending. This implies that the patch(es) on KorB with which KorA interacts is/are either present in multiple copies, on different faces of the KorB surface, or is/are in a sufficiently flexible position to allow multiple architectures for the interaction. Alternatively it may be that the KorB spreads from its operator by recruiting additional KorB dimers in such a way that they provide for flexible interaction. Since KorA can potentiate silencing by KorB even when its binding sites lies between the KorB binding site and the target for silencing **(reported in Chapter 4),** it seems likely that the higher order complexes that KorB can make with DNA and KorA are complex and therefore simple models for a tertiary Protein-Protein-DNA complex may not be applicable.

Tyrosine is often found to be involved in protein-protein interactions, either through the aromatic ring or the hydroxyl group. In transcriptional regulation a tyrosine residue in the C-terminal domain of the α subunit (α CTD) of RNA polymerase is critical for regulatory interaction with Spx, a global transcription regulator from *Bacillus subtilis* (Newberry et al., 2005), while Y8 in the elongation factor RfaH is critical for its interaction with the β' subunit of RNA polymerase (Sevostyanova et al., 2008). A dependence on multiple conserved tyrosine residues has also been observed in transcriptional activation by the EWS/ATF1 oncogene (Feng and Lee, 2001). In a different context, Y548 in the P16 domain of *Bacillus stearothermophilus* DnaG primase is critical for the interaction with DnaB helicase (Bailey et al., 2007; Chintakayala et al., 2008), but in this example it appears that the phenolic hydroxyl group is a critical part of the interaction, contacting the amide oxygen of N101 in the N-terminal region of DnaB. How the aromatic ring of Y84 interacts with KorB will therefore be of considerable interest.

General Discussion

RK2 is a broad host range plasmid. It encodes global transcriptional regulators, such as repressors KorA, KorB, KorC and TrbA. KorB plays a dual role in the biology of the plasmids. It is a DNA binding component (i.e. ParB homologue) of the RK2 active partitioning system as well as the regulatory protein of the major plasmid functions (replication, stable maintenance and conjugative transfer). KorB role as a regulatory protein has been explored in this study.

KorB can engage in pairwise cooperative interactions with KorA or TrbA, resulting in enhanced repression of transcription (Kostelidou et al., 1999; Zatyka et al., 2001). The synergy between KorB and KorA has been shown to be based on cooperative binding of DNA by this pair of proteins (Kostelidou et al., 1999). The N-terminus of KorA contains a predicted helix-turn helix domain and has predicted structural similarity to many other bacterial transcriptional regulators. The CTD of TrbA and KorA have been shown to be essential for cooperative interactions with KorB (Kostelidou et al., 1999; Zatyka et al., 2001) and it also has a role in dimerisation (Bhattacharyya and Figurski, 2001). Cooperative interaction between KorB and KorA / TrbA seems to involve a direct protein-protein interaction (Kostelidou et al., 1999; Zatyka et al., 2001, Bingle et al., 2008). KorA shares a conserved C-terminal domain (CTD) with TrbA repressor, and this domain is also present in the middle of protein KlcB (function unknown) (Jagura-Burdzy and Thomas, 1992; Larsen and Figurski, 1994). The obvious homology of the CTDs of KorA and TrbA suggests that this domain may be considered as a cooperativity “module”. In this study attempts have been made to elucidate the nature of interaction between KorB and DNA (i.e. DNA binding and gene silencing), KorB and KorA, and KorB and TrbA, and its mechanisms of repression from proximal and distal operator binding sites of KorB.

DNA binding studies of the deletion derivatives of KorB revealed that the N-terminus (up to 150 aa), C-terminus of KorB (up to 105 aa) and the linker region 255-285 aa are not essential for its interaction with DNA. KorB becomes defective in DNA binding by deletion of more than 20 aa upstream 255 aa of KorB. The DNA binding region of KorB lies between 151-230 aa (data not shown). This is consistent with the predicted HTH motif (K171-T190) (Motallebi-Veshareh et al., 1990), as well as with the crystallographic data for the internal part of KorB (173-252 aa) that showed that two helices, α3 (171-177 aa) and α4 (182-192 aa), connected by four residue turn from the HTH motif. The fact that monomeric KorB (deletion of 3 or more aa from the C-terminus makes KorB monomeric) binds DNA specifically indicates that dimerisation is not required for DNA binding. However, DNA binding affinity of monomeric KorB (i.e. C-terminal mutants) is much lower than that of dimeric WT KorB. On the other hand, removal of the N-terminus makes the KorB-DNA complexes more stable, which could be achieved in different ways. First, it could be that the N-terminus adds negative charge, which mean the electrostatic repulsion between KorB and DNA is greater (this could be indirect, possibly by neutralising the charges that help stabilise the KorB-DNA complex). Second, it could be that the N-terminus changes the conformation of KorB so that the position of the DNA binding motifs of the KorB dimer don't easily fit into the O_B operator and the DNA gets stressed or bent on binding to KorB. Third, it could be that KorB becomes more flexible since it can take up different conformations so that it doesn't need to stress DNA.

The repression experiments presented in this study demonstrate that deletion of the linker region (255-285 aa) and 30 aa from the N-terminal domain results in an extremely weak D-repression (distal repression obtained from O_B located at a distance from the promoter), which is restored in the presence of second global regulators i.e. TrbA or KorA. However, other deletions in KorB resulted in complete loss of repression and cooperativity at a distance. This also indicates that the ends of KorB are not necessary to interact with RNAP and other

regulatory proteins. Apart from the deletions made within 235-255 aa, all of the KorB mutants showed P-repression (proximal repression obtained from O_B located proximally to the promoter). The KorB mutants, which show P-repression on their own but need a partner protein to show D-repression, indicate that they might be defective in spreading but have the region to interact with TrbA/KorA, bound to the overlapping region of the promoter, via looping resulting in the KorB-KorA or KorB-TrbA complex, which would then be interacting with RNAP to repress the promoter. However, the rest of mutants, except those with deletions in the 235-255 aa region, show P-repression and P-cooperativity (interaction with KorA/TrbA from proximal O_B) but are defective in repression and cooperativity from distal O_B. This indicates that they might be defective in bending DNA to interact with TrbA and KorA, and thus can't reach RNAP to repress the promoter. KorB internal region 235-255 aa was investigated further by substitution mutagenesis.

Previous results showed that mutations at 240 aa (R240) into alanine (R240A) resulted in the loss of DNA binding (Kazimierczak et al., unpublished, Khare et al., 2004). However, the results obtained in this study show that this defect is eliminated when E237 and R240 are substituted to alanine to give KorB E237AR240A, which binds DNA stronger than WT. Therefore R240 is only essential when E237 is present and seems to counteract the charge of glutamate E237. This shows that negatively charged KorB (-21) doesn't bind DNA via positively charged residue; rather, the balance of charge in the internal region of KorB modulates this activity. This suggests that the role of R240 is to help attract KorB to the DNA and to counteract the repulsion between the backbone and the E237 side chain. The proposal that R240 was essential for operator recognition (Khare et al., 2004) was not consistent with the normal principles of bacterial gene regulation and it seems sensible at this stage at least to revise the conclusion. Nevertheless, the observations are not entirely negative because they suggest an alternative role for R240, that appears to be critical in allowing E237 to perform its

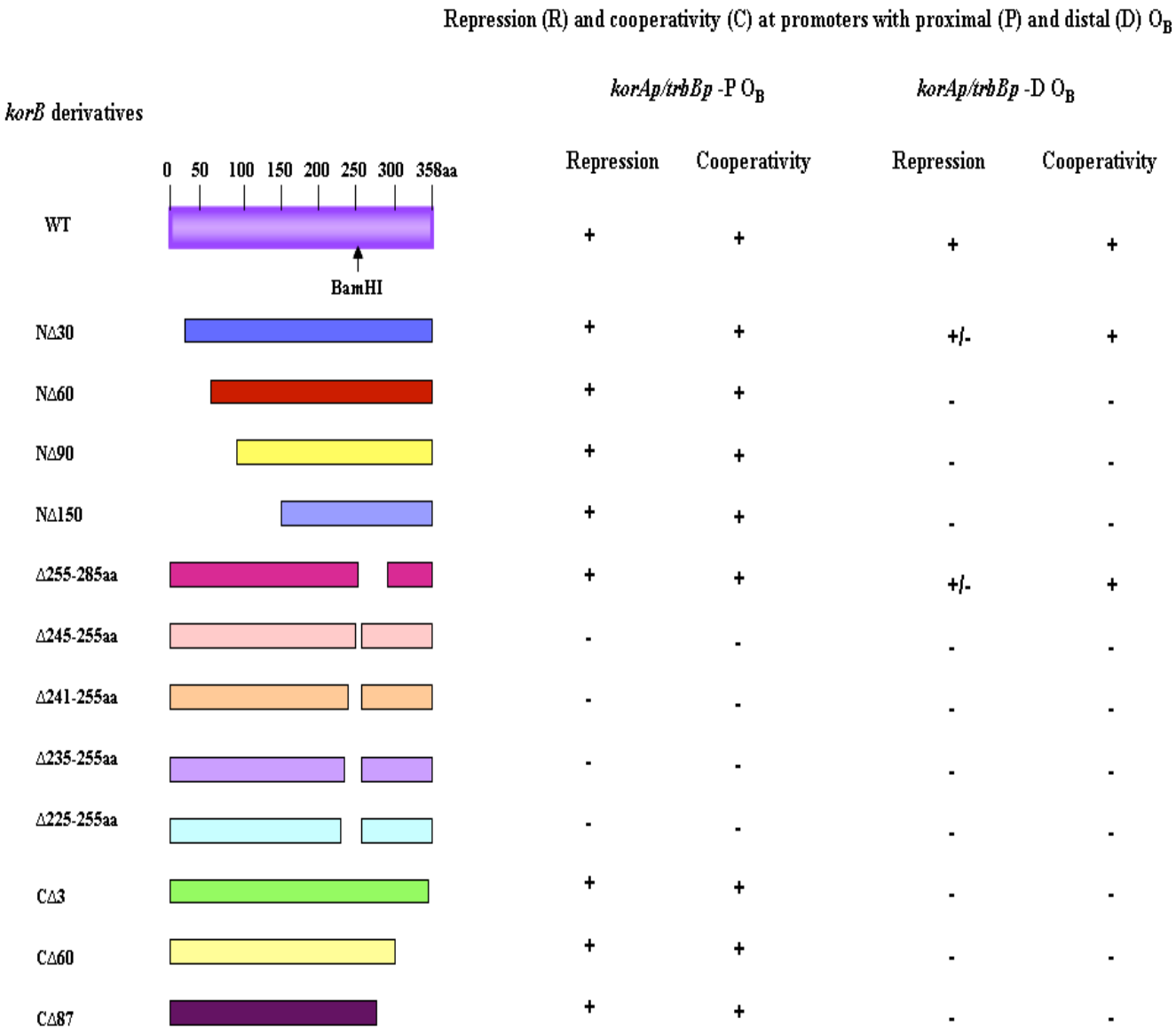


Figure 7.1: Summary of transcriptional repression and cooperativity activities of KorB (WT/deletion mutants) at *korAp* and *trbBp* with proximal and distal O_{Bs}. The + sign indicates proteins that can still repress and cooperate and – sign indicates the proteins which can't repress and cooperate, and +/- means weak in repression or cooperativity.

function. From the available crystal structure it appears that E237A sits close to the phosphate backbone and between the subunits of KorB that bind to the two halves of an operator. KorB E237 is located at the interface between KorB monomers. The KorB internal region clamps the operator and E237 creates electrostatic stress. It is known that KorB binding to DNA causes bending and results obtained in this study suggest that this can be assisted by the basic residues that help to grip the DNA on either side of the acid residues that tend to repel the DNA, thus acting as an electrostatic lever. KorB R240A, E237A, E237A R240A should be crystallized to link these results with the structural conformations. Given that KorB is a largely acidic protein, despite its DNA-binding region having a local basic character, it seems likely that a number of KorB dimers bind together to form a protein core around which DNA could wrap like a histone.

Repression data obtained with the substitution mutants show that KorB L246 is required for repression. KorB F249A represses and cooperates normally from proximal O_B but loose repression and cooperativity from distal O_B. Other KorB substitution mutants (E237, L231, L245 and L250) lose repression from distal O_B (-189 bp) on their own, which is restored in the presence of partner proteins i.e. KorA and TrbA. By increasing the distance between *trbBp* and O_B9 up to 1.5 kb, we showed that KorB NΔ30, Δ255-285 aa, E237A, L231A, L245A and L250A can still repress when the second global regulatory protein i.e. KorA or TrbA is present. This might be achieved either by looping between distant sites in a supercoiled molecule, or by spreading along DNA. However, the estimation of KorB concentration *in vivo* at approximately 500 dimers per *E. coli* cell (Balzer et al., 1992) argues against spreading as the mode of action for KorB from the distal O_B. Assuming five to ten copies of RK2 per cell, (Balzer et al., 1992) this allows an average of only four to eight dimers of KorB per operator. Thus understanding the ability of regulatory proteins to influence the activity of a promoter a long distance from their binding sites is of considerable

importance in modelling genome-wide expression. The IncP-1 plasmids represent an interesting model system because of the highly conserved binding sites for KorB protein, half of which are more than 500 bp from the nearest promoter. From the two known roles for KorB, these sites should be involved in either gene regulation or partitioning.

KorB substitution mutants (i.e. E237A, L231A, L245A, F249A, L250A and L246A) studied here had little or no effect on regulation or cooperativity at *trbBp* by changing the helical face of DNA to which KorB binds, at positions either distal or proximal to a promoter. This suggests that KorB is flexible in its regulatory interactions with promoter DNA/RNAP and with cooperating regulators, KorA or TrbA. However, both repression and cooperativity are unaffected by insertion of an additional 5 bp between the operators, which should move the relative positions of these proteins by 180^{o} (Bingle et al., 2005). It therefore seems unlikely that the proteins are relatively rigid shapes that interact simply by KorA- or KorB-induced DNA bending. This shows that the patch on KorB with which KorA interacts is either present in multiple copies, on different faces of the KorB surface, or is in a sufficiently flexible position to allow multiple architectures for the interaction. Alternatively it may be that the KorB spreads from its operator by recruiting additional KorB dimers in such a way that they provide for flexible interaction.

KorB operator binding sites are located either upstream or downstream of the promoter in RK2, whereas KorA and TrbA operator binding sites are present in the promoter region. KorB alone is a weak repressor compared to KorA/TrbA, which could be due to the differences in the location of binding sites. To rule out this possibility, an artificial promoter *trfAp-1* was created with KorB binding site overlapping the -10 region of the promoter. The results show that KorB binding to the region overlapping the promoter doesn't improve its repression. This indicates that KorB binding to the O_B is not sufficient for transcriptional repression.

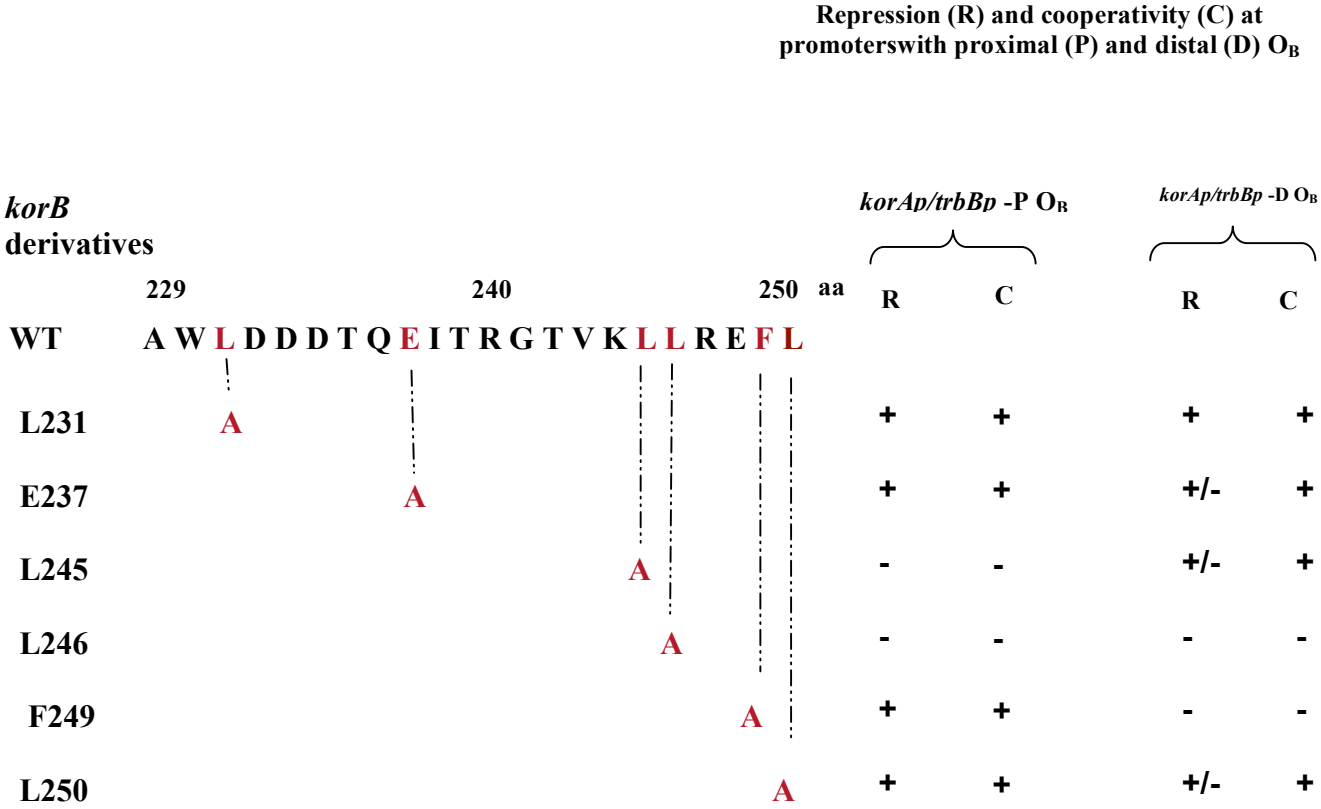


Figure 7.2: Summary of transcriptional repression and cooperativity activities of KorB (WT/substitution mutants) at *korAp* and *trbBp* with proximal and distal O_{Bs}. The + sign indicates the derivatives can still repress or cooperate, and – sign indicates the derivatives which can't repress or and cooperate, and +/- sign indicates very weak repression or cooperativity.

Therefore, KorB must actively interfere with RNAP activity once it has bound to the DNA (as we concluded from detailed studies at the *korAp*) and tends to contradict the observation that KorB excludes RNAP from the *trfAp* – unless KorB can somehow bind to similarly positioned O_B sites in different ways so as to exclude RNAP in some contexts but not others.

The results presented here show that KorB is very flexible in its modes of repression. For example, KorB can interact with KorA, TrbA and RNAP with sufficient flexibility to operate between molecules on either the same or opposing faces of the DNA strand. KorB might achieve this via a direct but highly flexible protein–protein interaction between the proteins or via an effect of KorB on local DNA topology, which would then affect promoter melting and/or binding of KorA or TrbA. Recent KorB structural studies have indicated that the C-terminal dimerisation domain, which holds the monomers together in solution, is joined to the DNA-binding domain by a putative flexible linker (Khare et al., 2004; Delbruck et al., 2002). There is a second, N-terminal, dimerisation region that can function in the KorB complex with DNA, so that an operator may accommodate two dimers, each with a free monomer that could either promote spreading by attracting other KorB molecules, or contact RNAP or a second repressor (Lukaszewicz et al., 2002; Leonard et al., 2004). In conclusion, the flexibility of KorB interaction with other repressor proteins and RNAP leading to gene regulation is highly unusual in bacteria. The only examples of which we are aware come from eukaryotic systems, in particular the Gal4 activator, where DNA looping in combination with a DNA flexibility-enhancing action of high mobility group (HMG) proteins allows for activation from a wide range of positions (Ross et al., 2001). This study shows that mutations in KorB can have differential effects on its ability to loop or spread along DNA, and therefore on its repression.

A number of ParB proteins (i.e. P1) are able to silence genetic functions at a distance in a rather non-specific manner. This is thought to occur by spreading along the DNA from a nucleation site. Gene silencing by KorB has been formally reported for the first time by this study. The expression of KorB *in trans* to the pSC101 replicon plasmid carrying the O_B site was shown to cause plasmid loss, irrespective of the orientation of the inserted O_B-carrying fragment. Simultaneous expression of KorA or TrbA did not act as a roadblock to KorB silencing/spreading, irrespective of the direction. Rather, the presence of KorA or TrbA potentiate the KorB silencing/spreading, which reinforces the possibility that they may actually promote the recruitment of KorB to, and spreading from, particular sites. Deletion of 30 aa from the N-terminus and the linker region did not affect the ability of KorB to silence gene, and the same effect was observed at a lower concentration of KorB in the presence of KorA or TrbA. However, other deletion mutations in KorB resulted in the complete loss of this ability and the presence of KorA or TrbA could not potentiate it. This shows that full-length KorB, except 30 aa from N-terminus and the linker region, is required to show D-repression and gene silencing/spreading.

Mutation E237A has very interesting effects on the biological activity of KorB. On its own, this protein has lost the ability to act at a distance – it does not repress *trbBp* through O_B9 or *trbAp* and does not cause silencing. This suggests that the protein is defective in spreading along the DNA away from an O_B site. The footprint tends to fit with idea of less spreading (data not shown). In short, KorB E237A (which is defective in silencing) cannot repress at a distance alone, but can do so in presence of TrbA – strong evidence of looping. The fact that KorA and TrbA do not potentiate gene silencing by E237A, but do potentiate its repression, indicates strongly that gene silencing is caused by spreading instead of looping. Mechanisms of KorB distal repression have been shown in the form of a model in **Figure 7.3.**

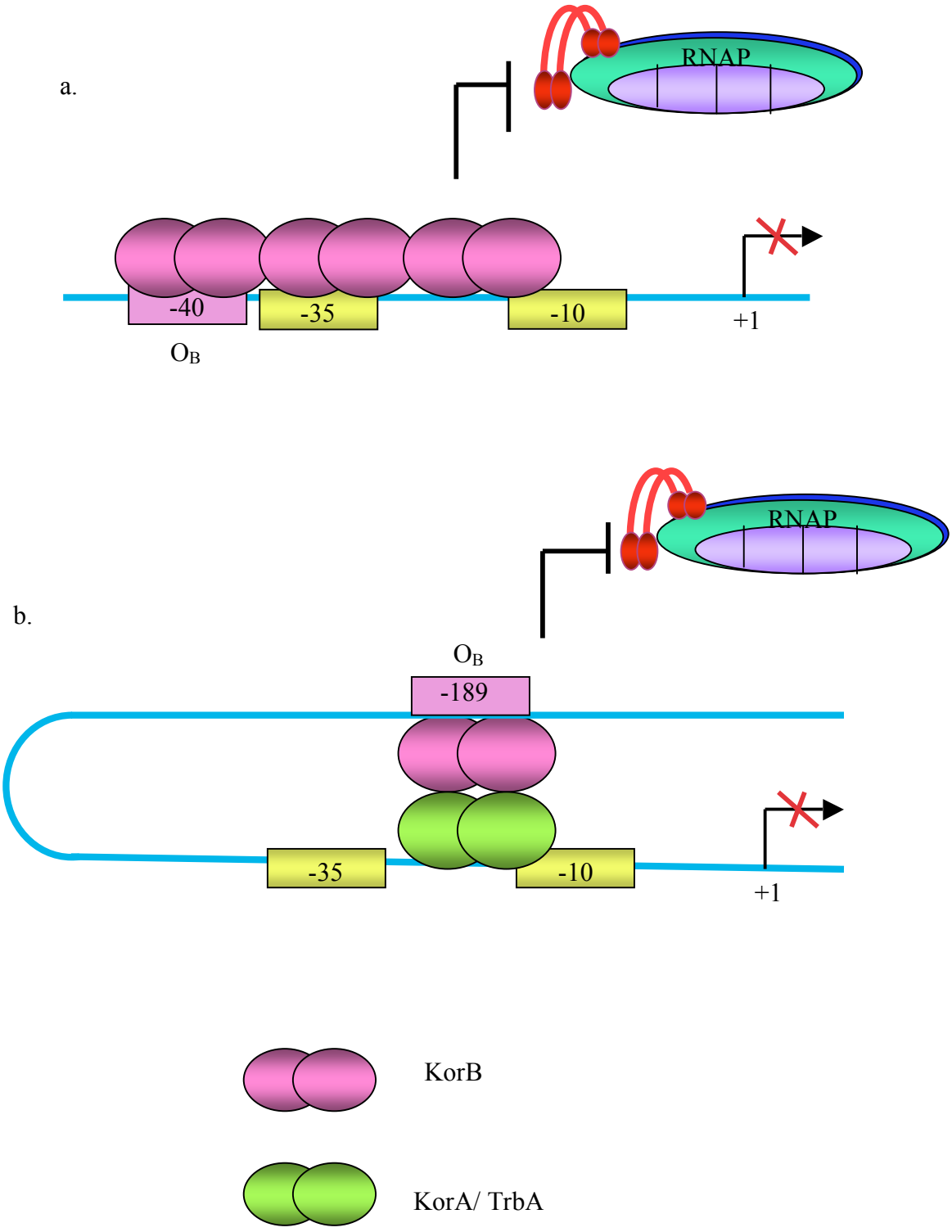


Figure 7.3: Model describing mechanisms of KorB repression from proximal and distal O_B. (a) shows KorB repression from proximal O_B (KorB P-repression) in the absence of any second global regulator. KorB binds to the operator site close to the promoter and does not allow RNAP to bind DNA or otherwise excludes RNAP from binding the promoter. (b) shows KorB repression from distal O_B (KorB D-repression). If KorB becomes defective in spreading, it can still repress from distal binding sites by by interaction with other global regulators bound at the promoter.

KorA can interact with KorB *in vitro* in the presence and absence of DNA having O_B (irrespective of the presence of O_A), as shown by electromobility shift assay (EMSA) and protein co-purification assays. KorA potentiation of KorB binding to a DNA fragment containing O_B was reduced by the Y84A mutation in the C-terminus of KorA as shown by *in vitro* and *in vivo*. Given the dual role that this region of KorA appears to play (i.e. dimerisation and cooperativity), we may be fortunate in finding a residue that only appears to affect one of the two major functions and that seems to have such a marked effect on cooperativity, since a degree of individual redundancy has been observed in the amino acids involved in other protein-protein interfaces (Jobichen et al., 2007). It is likely to be the aromatic ring of tyrosine that is critical for the interaction with KorB. It could be that Y84 protrudes from KorA so that it can contact a suitable part of the surface of KorB as shown in **Figure 7.4**.

Since KorA can potentiate silencing by KorB even when its binding site lies between the KorB binding site and the target for silencing, it seems likely that the higher order complexes that KorB can make with DNA and KorA are complex, and therefore simple models for a tertiary Protein-Protein-DNA complex may not be applicable. Experiments currently underway are exploring the consequences for the plasmid of destroying this cooperative interaction to determine just how advantageous this cooperativity is for the survival and competitiveness of the plasmid.

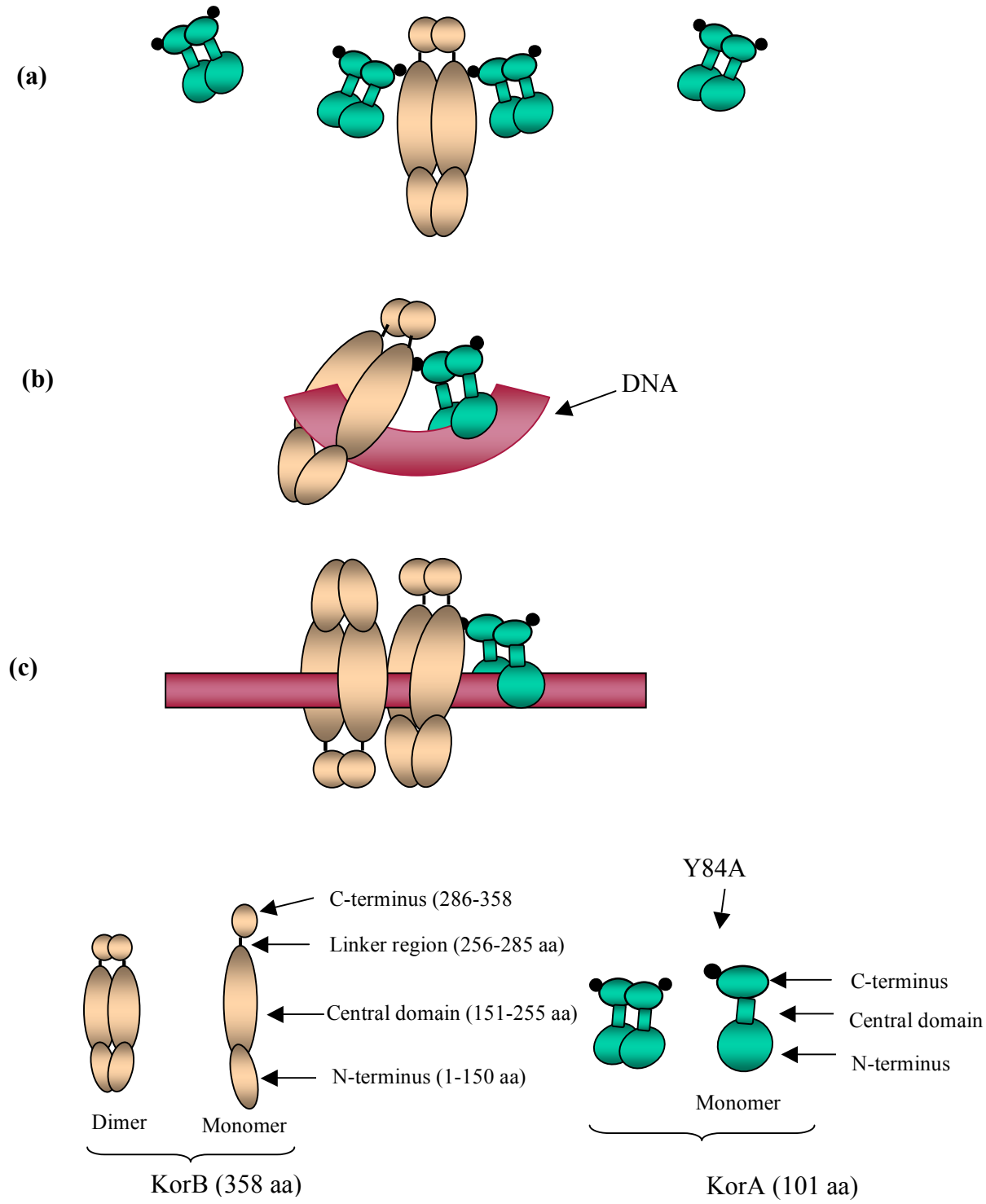


Figure 7.4: Scheme to summarise possible KorB-KorA interactions. For KorB, HTH is located in the central domain and for KorA it is present in the N-terminus. (a) KorB can interact with KorA in solution as shown by protein co-purification assays. However, on DNA the presence of specific binding sites constrains the location of adjacent KorA and KorB dimers and contact may be made by looping (b) or spreading (c) in a flexible way since rotation of the binding sites through 180^{o} to each other does not interfere with observed cooperativity (Bingle et al., 2005).

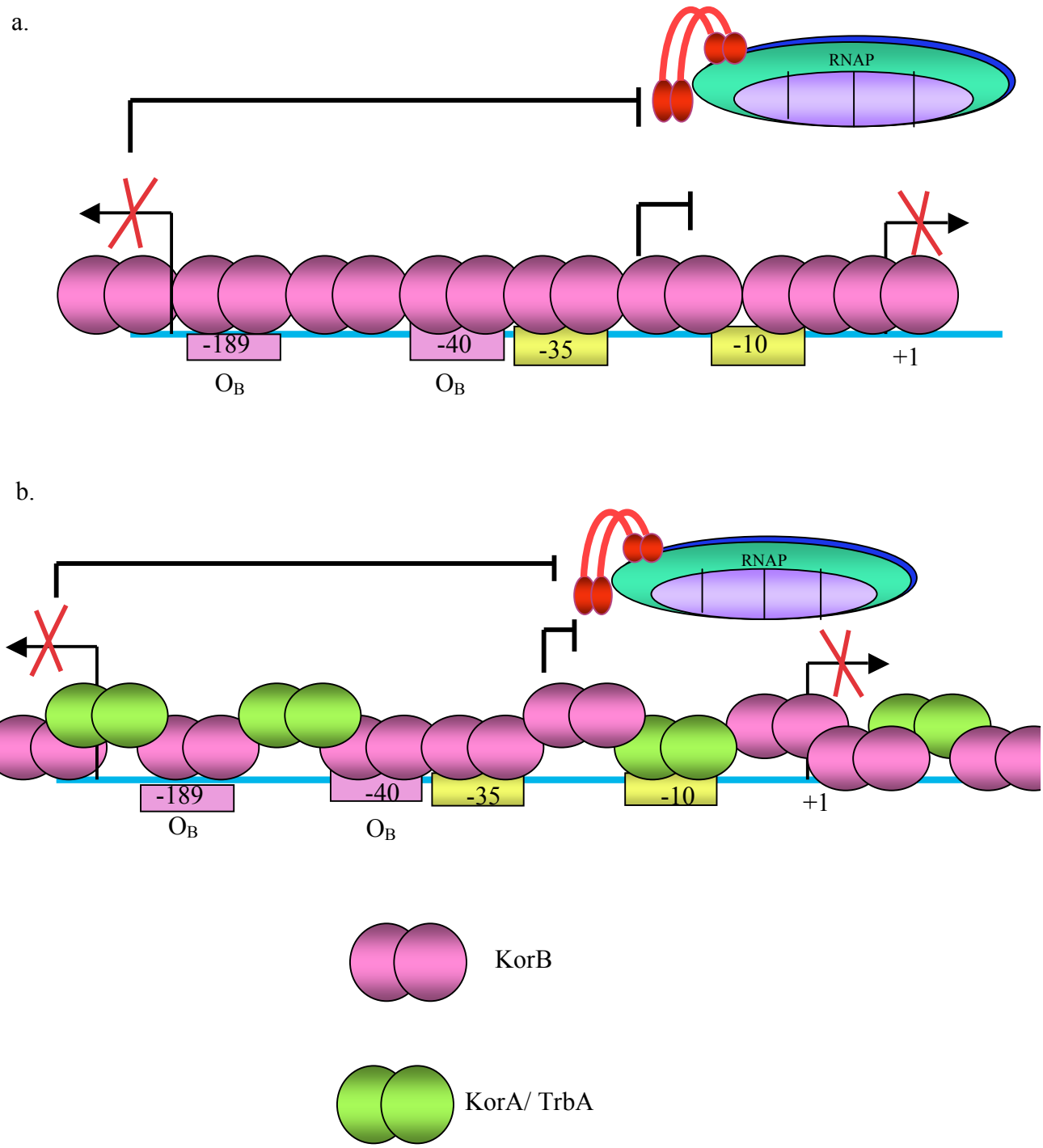


Figure 7.5: Model for KorB gene silencing. (a) KorB binds specifically to the operator and spreads along DNA in a sequence independent manner. This leads to interfere with the expression of neighbouring genes. TrbA and KorA potentiate the silencing activity of KorB. (b) In the presence of KorA or TrbA, gene silencing is achieved at lower KorB concentration. This shows that KorA and TrbA do not act as roadblock in gene silencing by KorB.

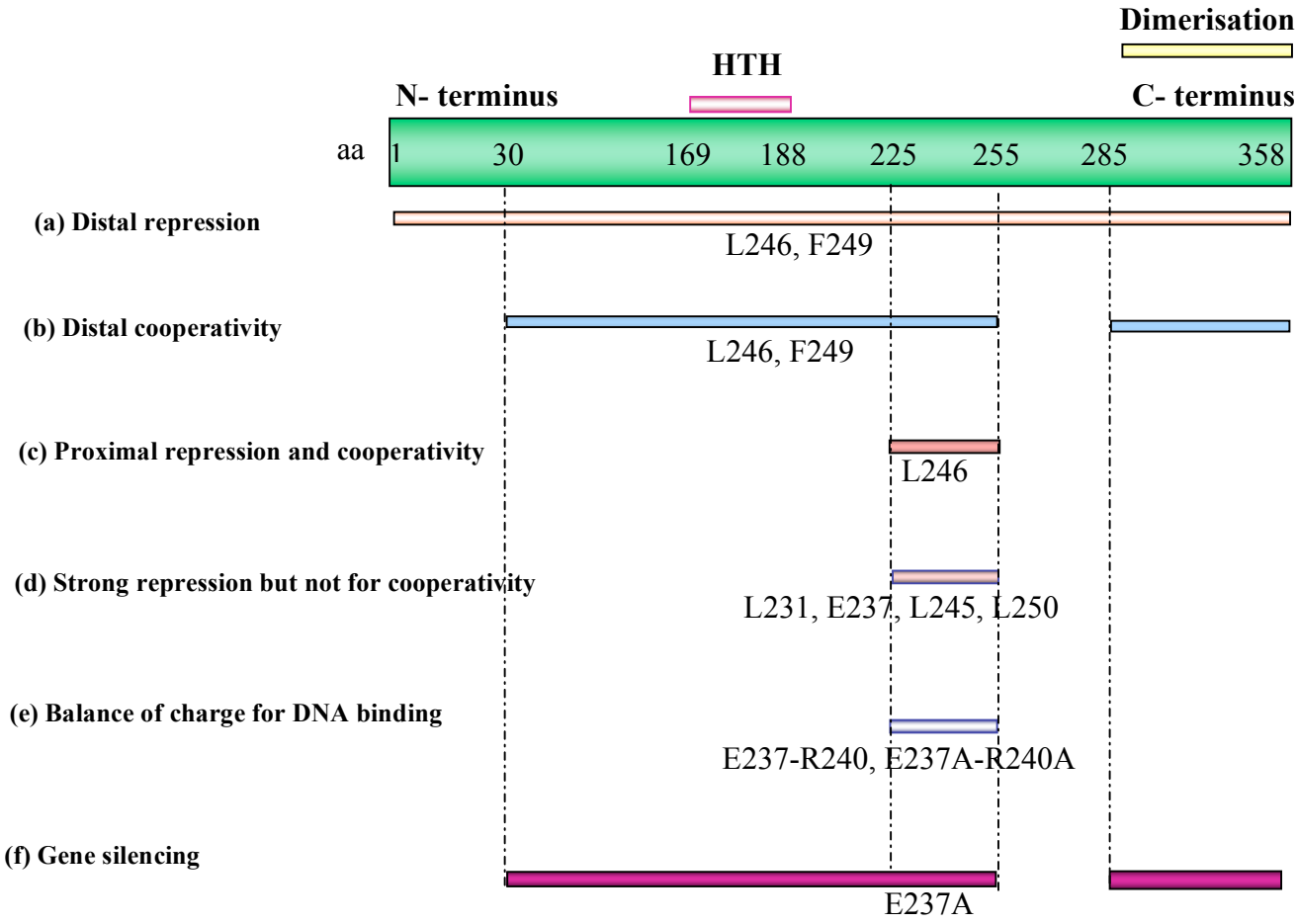


Figure 7.6: Schematically representation of functions assigned to the different domains of KorB, based on deletion and substitution mutagenesis of KorB followed by biochemical characterisation. **(a)** Full length KorB is required to repress from distal O_B. KorB L246A and F249A completely lose the ability to repress from distal O_B. **(b)** 30 aa from N-terminus and the linker region (255-285 aa) is not required by KorB to cooperate with KorA or TrbA to achieve better repression of the promoter from distal O_B. **(c)** The region 225-255 aa of KorB is essential for repression as well as cooperativity irrespective of the location of O_B to the promoter. By changing the residue L246 into alanine (A), KorB results in the complete loss of repression and cooperativity at proximal as well as distal O_B. **(d)** KorB L231, E237, L245, L250 are essential to achieve normal repression from distal O_B. However, they can restore their ability to repress strongly by cooperating with KorA and TrbA from distal O_B. **(e)** Balance of change in the internal region 225-255 aa of KorB is required for DNA binding. Mutation of glutamate E237 into alanine and the double mutant of E237A with R240A bind DNA stronger than WT. This shows that R240 is essential only when E237 is present and they seem to counteract the charge of glutamate E237. **(f)** Apart from 30 aa from N-terminus and the linker region, all regions of KorB are essential for its gene silencing ability. KorA and TrbA potentiate gene silencing by KorB. On the other hand, KorB mutants defective in gene silencing can not restore it even in the presence of KorA and TrbA. This study also shows that KorB mutants defective in repression at a distance are also defective in gene silencing and KorA and TrbA can not restore it. This contrasts the situations where KorA or TrbA can restore the defect of distal repression- shows that distal repression is restored via looping the DNA and that gene silencing is linked with KorB ability to spread along DNA.

Figure 7.6 shows a model that is summarising most of the functions of KorB explored in this study using deletion and substitution mutagenesis. This study has helped to identify the domains of KorB related to different functions i.e. repression, cooperativity, protein-protein interaction, protein-DNA interactions. Specific mechanisms and the residues critical for repression and cooperativity from distal and proximal O_B have also been determined. Gene silencing by KorB has been reported for the first time and the role of KorA and TrbA in gene silencing has also been explored. Further work is required based on these findings to explore the additional functions of the domains of KorB. KorB spreading and looping should be tested further using electron microscopy using WT and E237A mutant, which is defective in spreading and gene silencing. KorB (WT and interesting mutants like E237A) should be tested for its ability to bend DNA in the presence and absence of partner proteins i.e. KorA/TrbA using linear dichroism. KorB complexes with DNA, RNAP and KorA/TrbA should be studies further using footprinting at both proximal and distal promoters i.e. *korA* and *trbB* promoters to identify that region and residues that interact with RNAP and also to study in detail the mechanism of repression at different promoters. KorB should be compared for its binding to linear and supercoiled DNA to find its role in DNA topology and supercoiling. Thus, additional analysis proposed in this study would help further broaden the understanding of the composite process of gene regulation.

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